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# SOME FACTORS ASSOCIATED WITH THE BREEDING OF ANOPHELES MOSQUITOES[1]

By G. H. BRADLEY

*Associate Entomologist, Division of Insects Affecting Man and Animals, Bureau of Entomology, United States Department of Agriculture*

## INTRODUCTION

The fact that in a given locality Anopheles is found breeding in certain water areas and not in others, or sparsely in some areas and abundantly in others, has given rise to considerable conjecture and no little investigation as to the causes of this condition. The present paper summarizes the results of two seasons' observations in the vicinity of Mound, La. This locality is in the northeastern part of Louisiana in what is known as the Louisiana Delta region. Here the land is more or less flat, which accounts for the poor natural drainage and gives rise to numerous shallow lakes in the lower areas. All of these areas are, to a greater or less extent, covered by tree growths. The streams of the region are known as bayous. They are sluggish, having little or no current except after heavy rains, and are usually more or less overgrown with trees and brush, except in such stretches as have been cleared of this material. Land-locked branches of bayous form sloughs which are similar to the bayous except that they are stagnant throughout the year and usually contain larger quantities of aquatic and semiaquatic vegetation. These areas, all of which produce Anopheles to a greater or less extent, have been described in a previous paper (*3*).[2]

## METHODS OF OBSERVATION

Observations on the occurrence and abundance of Anopheles at selected points, or "stations," in these water areas were made monthly from May to September, inclusive, during 1928 and 1929. The larval abundance rate was determined on the basis of the number of larvae taken in a collection of 10 dips of surface water, the dips being made with an ordinary white-enameled water dipper about 5 inches in diameter. At the time of each collection observations were made on the environmental conditions in the area, and the hydrogen-ion concentration of the surface water was determined. A sample was taken of the top one-half inch of the water and this was carried immediately to the laboratory, where, after thorough shaking, 1 c c was placed in a Sedgwick-Rafter counting cell and a count made of the number of organisms in 40 c mm of water, as a basis for computing the rate of occurrence of the organisms per cubic centimeter in the water. After this count the organisms in a large

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[2] Reference is made by number (italic) to Literature Cited, p. 399.

sample of the water were concentrated by either filtering or centrifuging and the sediment was examined for the presence of other forms. Samples of algal patches and algae-coated sticks were also collected and examined.

In these examinations determinations, as far as genera when possible, were made of the forms observed, but those not readily fitting into the keys available were grouped only into classes. The Anopheles larvae collected were taken, in water from the breeding area, to the laboratory, determined as to species, and dissections made of some, usually five, of the larger specimens in order to ascertain the nature of the food ingested. In only two instances were species other than *quadrimaculatus* found, and these were taken from areas where *quadrimaculatus* also occurred. In these two cases the gut contents were similar for all species.

## INFLUENCE OF TEMPERATURE, PRECIPITATION, AND QUANTITY OF SURFACE WATER UPON ANOPHELES LARVAL ABUNDANCE RATES AND PLANKTON CONTENT OF THE WATER

In summarizing the observations it has been assumed that temperature and other conditions influencing the activity of Anopheles during the five months from May to September, inclusive, are sufficiently constant to make all observations comparable. Table 1 gives a summary of the larval collection records and plankton counts for the two seasons, arranged by months, together with pertinent meteorological data and a surface-water index. This index is the average of the width in feet of the water area at the various observation stations, computed or measured when the larval collections were made. The records on plankton findings for each year are divided into two groups—those for the breeding stations where Anopheles usually were found, and those at stations located in impounded bayou areas where, on account of special conditions, Anopheles larvae were consistently absent.

The figures for 1928 show the monthly larval rate as having a seasonal rise and fall with the rise and fall of the mean air temperature, the highest rate being in August, when the highest mean temperature occurred. For the following year, however, there are two peaks in the larval rate, one in May and the other in September, in which months the lowest mean temperatures occurred. The writer has found from observations in this locality extending over a number of years that the variation in summer temperatures does not noticeably affect larval population as measured by dipping. In a previous paper (*3*) the larval rates obtained during three other seasons are given and further illustrate this fact.

The summer rainfall in this region is not usually sufficient to keep the surface-water areas from showing a steady decrease as the season advances. This gradual decrease in water surface tends to keep the margins of the water areas more or less free from vegetation, which encroaches on the shore line in times of stationary or rising water, and to remove from the surface of the water large quantities of flotage which are blown to the margins by the wind. This is not refloated, except in case of exceptionally heavy rains, until the high waters of the ensuing winter. (Fig. 1.) The lowering of the water level also gradually leaves the tree and brush covered marginal areas, particularly those of the lakes, dry; and in the lakes the central parts

of which are devoid of aquatic vegetation the production of Anopheles ceases.

In the data given in Table 1 it is seen that the surface-water index for 1928 fell only gradually from May to August and was accom-

FIGURE 1.—An Anopheles breeding place at the edge of a swamp lake. Note how the débris is becoming stranded as the water level grows lower

panied by a rising larval rate. A rapid diminution of the water surface then occurred from August to September, attended by a falling larval rate. In 1929 the surface water decreased rather rapidly from May to August, as did the larval abundance rate. From August to

September, however, there was little fall in the surface water, making for more or less stable conditions in the breeding areas, accompanied by a rise in the larval index. These conclusions are of course applicable only to conditions as they occurred in this locality during the two years under consideration, for it is known that under certain conditions the opposite may be true, that is, a lowering water level may increase larval abundance by making large central open areas in lakes suitable for the breeding of Anopheles. One example may be cited of a large lake in which the early spring breeding was entirely marginal. The bed of this lake was almost completely covered by a thick growth of Potamogeton and Chara, which early in the season did not reach the surface. As the water became shallow, the long stems of these plants came to lie just at the water surface, providing excellent protection for Anopheles larvae and giving rise to the most intensive breeding over hundreds of acres of water surface.

TABLE 1.—*Larval rates of Anopheles and plankton counts compared to temperature, precipitation, and surface water index at Mound, La.*

(One collection of 10 dips made at each station each month)

BREEDING STATIONS

| Year and month | Number of stations | 10-dip larval rate | Precipitation | Surface water index | Temperatures | | | Average number of organisms per cubic centimeter | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Maximum | Minimum | Mean | Total plankton | Flagellates | Ciliates | Amoebids | Diatoms | Chlorophyceae | Cyanophyceae | Miscellaneous |
| 1928 | | | *Inches* | | ° *F.* | ° *F.* | ° *F.* | | | | | | | | |
| May | 13 | 4.2 | 2.67 | 340 | 82.3 | 59.0 | 70.7 | 5,159 | 4,602 | 119 | 3 | 294 | 141 | 0 | 0 |
| June | 13 | 8.75 | 8.94 | 310 | 86.6 | 68.9 | 77.8 | 9,025 | 4,350 | 450 | 8 | 909 | 3,208 | 96 | 4 |
| July | 12 | 12.4 | 5.25 | 274 | 91.3 | 70.7 | 81.0 | 5,535<br>[a]43,356 | 4,102 | 256 | 2 | 865 | 225 | 23<br>[a]36,894 | 62 |
| August | 13 | 14.1 | 1.90 | 248 | 93.4 | 71.1 | 82.3 | 11,700 | 7,444 | 654 | 123 | 1,873 | 1,177 | 175 | 254 |
| September | 10 | 6.4 | .89 | 129 | 83.6 | 58.9 | 71.1 | 14,135 | 8,300 | 612 | 58 | 3,430 | 977 | 175 | 583 |
| 1929 | | | | | | | | | | | | | | | |
| May | 12 | 15.4 | 2.83 | 362 | 82.2 | 62.1 | 72.2 | 4,527 | 3,713 | 129 | 4 | 383 | 277 | 13 | 8 |
| June | 12 | 11.8 | 2.76 | 323 | 89.2 | 66.7 | 77.9 | 7,415 | 5,389 | 117 | 48 | 1,569 | 219 | 69 | 4 |
| July | 11 | 10.1 | 4.51 | 244 | 92.2 | 69.8 | 81.0 | 9,432 | 4,450 | 384 | 27 | 1,298 | 1,641 | 1,562 | 70 |
| August | 6 | 6.4 | 1.82 | 123 | 93.2 | 66.6 | 79.9 | 16,164<br>[a]66,031 | 8,575 | 275 | 18 | 1,432 | 5,550<br>[a]56,397 | 193 | 121 |
| September | 4 | 14.0 | 1.10 | 107 | 88.6 | 63.1 | 75.8 | 11,069 | 3,238 | 287 | 0 | 1,082 | 5,287 | 1,150 | 25 |

IMPOUNDED BAYOU STATIONS

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1928 | | | | | | | | | | | | | | | |
| May | 3 | 0 | | | | | | 10,483 | 10,233 | 100 | 0 | 100 | 50 | 0 | 0 |
| June | 3 | 0 | | | | | | 15,283 | 4,883 | 450 | 0 | 1,292 | 7,667 | 991 | 0 |
| July | 3 | 0 | | | | | | 7,917 | 5,359 | 583 | 0 | 800 | 1,133 | 42 | 0 |
| August | 3 | 0 | | | | | | 9,867 | 6,567 | 600 | 0 | 1,183 | 1,317 | 200 | 0 |
| September | 3 | 0 | | | | | | 15,683 | 4,725 | 1,683 | 0 | 5,992 | 2,483 | 350 | 450 |
| 1929 | | | | | | | | | | | | | | | |
| May | 3 | 0 | | | | | | 2,525 | 1,616 | 167 | 17 | 550 | 133 | 42 | 0 |
| June | 3 | 0 | | | | | | 24,925 | 11,559 | 483 | 0 | 11,400 | 975 | 508 | 0 |
| July | 2 | 0 | | | | | | 6,387<br>[a]514,442 | 5,513 | 238 | 12 | 612 | 0 | [a]500,008 | 0 |
| August | 3 | 0 | | | | | | 25,642 | 5,675 | 433 | 0 | 975 | 3,609 | 14,900 | 50 |
| September | 2 | 0 | | | | | | 9,800<br>[a]461,000 | 5,950 | 425 | 0 | 925 | 2,425 | 75<br>[a]450,050 | 0 |

[a] Including the count from 1 collection which contained an extremely large number of 1 group of organisms.

Considering next the effect of temperature, surface water, and rainfall on plankton organisms (Table 1), it appears that there is a wide range of variation when the data for a like group of stations for the two years or for unlike groups during the same year are considered. The impounded-bayou group of stations in both years shows as a rule greater abundance of plankton than is shown by the breeding group. (Tables 1 and 2.) This condition is associated with clear water surfaces and absence of shade. (Fig.2.) An increasingly greater

FIGURE 2.—View along a cleared and impounded bayou, showing absence of débris and vegetation. Such places as this are mosquito-free

number of plankton organisms appears to be present in the water as the season advances and as the volume of water diminishes, but this increase is quite irregular and is caused no doubt by the common phenomenon of periodicity in certain species of organisms. On four different occasions one collection was found to contain so large a number of one kind of organism that it colored the entire body of water at the station where it was taken. The high counts obtained in these instances unduly affect the entire average and for comparative purposes are better omitted. The averages obtained when these high counts are included are shown in braces in Tables 1 and 2.

TABLE 2.—*Average number of plankton organisms as compared to larval abundance rates of Anopheles at Mound, La.*

| Year and number of observations | Larval abundance per 10-dip collection | | Average number of organisms per cubic centimeter | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Range | Rate | Total plankton | Flagellates | Ciliates | Amoeboids | Diatoms | Chlorophyceae | Cyanophyceae | Miscellaneous |
| 1928 | | | | | | | | | | |
| 15 | (a) | 0 | 11,847 | 6,354 | 683 | 0 | 1,873 | 2,530 | 317 | 90 |
| 10 | 0 | 0 | 10,927 | 7,302 | 532 | 25 | 2,174 | 574 | 205 | 115 |
| 16 | 1–5 | 2.13 | 8,407<br>b 37,160 | 4,500 | 458 | 17 | 736 | 2,552 | 25<br>b 28,221 | 119 |
| 13 | 6–10 | 7.46 | 8,535 | 6,194 | 271 | 37 | 929 | 706 | 94 | 304 |
| 9 | 11–15 | 13.33 | 6,672 | 4,511 | 328 | 47 | 1,203 | 355 | 61 | 167 |
| 3 | 16–20 | 18.67 | 12,592 | 10,625 | 633 | 17 | 642 | 567 | 108 | 0 |
| 6 | 21–25 | 23.50 | 10,129 | 4,154 | 400 | 146 | 3,712 | 1,367 | 167 | 183 |
| 3 | 26–30 | 28.30 | 9,275 | 6,650 | 433 | 0 | 1,284 | 800 | 0 | 108 |
| 1 | 31–35 | 33.00 | 3,100 | 2,475 | 375 | 0 | 200 | 50 | 0 | 0 |
| 1929 | | | | | | | | | | |
| 13 | (a) | 0 | 14,742<br>c 205,707 | 6,113 | 352 | 6 | 3,219 | 1,461 | 3,579<br>c 193,102 | 12 |
| 9 | 0 | | 17,975 | 11,028 | 383 | 14 | 1,211 | 4,103 | 1,236 | |
| 9 | 1–5 | 3.22 | 7,819<br>b 48,548 | 2,555 | 292 | 8 | 1,578 | 3,014<br>b 43,945 | 361 | 11 |
| 8 | 6–10 | 7.60 | 5,812 | 3,125 | 87 | 40 | 2,050 | 266 | 141 | 103 |
| 9 | 11–15 | 13.00 | 8,888 | 6,488 | 255 | 14 | 639 | 1,275 | 192 | 25 |
| 1 | 16–20 | 19.00 | 2,725 | 1,975 | 0 | 200 | 550 | 0 | 0 | 0 |
| 3 | 21–25 | 24.00 | 5,517 | 4,059 | 92 | 0 | 558 | 92 | 683 | 33 |
| 2 | 26–30 | 28.00 | 2,963 | 1,125 | 63 | 0 | 537 | 1,000 | 238 | 0 |
| 1 | 31–35 | 32.00 | 4,100 | 950 | 25 | 0 | 325 | 1,925 | 300 | 575 |
| 2 | 36–40 | 38.00 | 4,838 | 1,238 | 150 | 100 | 325 | 1,000 | 2,025 | 0 |
| 2 | 41–45 | 42.00 | 3,400 | 2,838 | 225 | 0 | 262 | 75 | 0 | 0 |
| COMPARISON OF LOW WITH HIGH BREEDING STATIONS (ONE COLLECTION OMITTED EACH YEAR) | | | | | | | | | | |
| 1928 | | | | | | | | | | |
| 29 | 1–10 | 4.55 | 8,465 | 5,260 | 374 | 26 | 822 | 1,725 | 56 | 202 |
| 22 | 11–35 | 19.70 | 8,616 | 5,448 | 406 | 61 | 1,776 | 707 | 85 | 133 |
| 1929 | | | | | | | | | | |
| 17 | 1–10 | 5.30 | 6,875 | 2,824 | 196 | 24 | 1,800 | 1,720 | 257 | 54 |
| 20 | 11–45 | 22.80 | 6,289 | 4,195 | 174 | 27 | 527 | 891 | 430 | 45 |

a Impounded bayou. b Including 1 high count. c Including 2 high counts.

## COMPARISON OF LARVAL RATES AND PLANKTON COUNTS

Table 2 gives a summary of the results of the organism counts for the two seasons under consideration, as compared to larval population, irrespective of environmental conditions in the breeding areas. The data are divided into three groups, viz, those made in the cleared and impounded-bayou areas where Anopheles was consistently absent; those made in areas where Anopheles was usually found but for some reason was temporarily absent; and those made in areas where Anopheles was present. The observations in the last group are divided into subgroups based on increasing larval population.

In addition to showing the general tendency for the nonbreeding waters to have a higher plankton content than the breeding waters, the data given in this table seem to indicate that among the breeding stations those having the greatest larval population have the lowest plankton population, this being true whether the total plankton or a major class of organisms is considered. By summarizing these records further, however, and making only two subgroups of breeding stations, viz, those with low larval rates (1–10) and those with high

larval rates (11 plus), it is seen that there is little difference in the average plankton population per cubic centimeter of these two groups when the total plankton is considered. In the individual classes of organisms the major differences in the rates of occurrence are not constant when one year is compared with another except in the case of the Chlorophyceae, and here, by the elimination of an additional high record from the low breeding group for each year, this apparent constant difference may be eliminated. (The average of 1,725 in 1928 becomes 439, and the average of 1,720 in 1929 becomes, 1,111.)

While it might appear logical to conclude that the absence of feeding by the larvae was responsible for the higher plankton rates in the nonbreeding groups of stations, it is believed that the environmental conditions prevalent in these areas were the principal cause of the large plankton population. In the impounded bayous the water is unshaded and has no aquatic vegetation to cover its surface. This condition, of course, favors more rapid growth and multiplication of the chlorophyll-bearing organisms than occurs in shaded or partly shaded areas in which most of the Anopheles breeding takes place hereabout. The group of observations made when Anopheles were temporararily absent also shows a high plankton count in each year. This may be explained by the fact that a part of the observations in this group were made at stations where the water had receded from marginal shaded areas in which larval protection had occurred and, at the time of observation, presented conditions similar to those in the cleared and impounded bayou areas previously mentioned.

## RATE OF OCCURRENCE OF THE FOUR COMMONEST PLANKTON GENERA IN ANOPHELES BREEDING AND NONBREEDING WATERS

In comparing the records on the rate of occurrence per cubic centimeter of the more common plankton genera with the absence of and the presence in increasing numbers of Anopheles larvae, no definite trends were found. The data in Table 3 illustrate this condition as it occurred in four of the most common genera of the flagellates. In this table the findings are grouped into the same divisions in regard to larval findings as are used in the latter part of Table 2. The average numbers of these genera per cubic centimeter are given and also the percentage of the total flagellate population of the water that these four genera represent. The data show that these genera are present in abundance in each of the groups of observations and that they compose practically the same percentage of the total flagellate population in each group, with the exception of a low percentage in the impounded-bayou group of stations in 1929. This is caused by larger counts of less common genera and not by any lack of abundance of the genera under consideration. It is also noted that the temporary nonbreeding-station group in each year had a high flagellate population, and this may be explained by the fact that some of these areas are bodies of water having very little shade and are sometimes more or less fouled by the wallowing of animals. This type of location has been found to be particularly suitable for the development of large numbers of flagellates, particularly *Euglena* spp., which often become so abundant as to color the water green.

TABLE 3.—*Occurrence of the four commonest flagellate genera, compared to Anopheles larval abundance, Mound, La., 1928 and 1929*

| Year and station group | Observations | Larval range | Average per 10-dip collection | Average rate per cubic centimeter | | | | | | Percentage of total flagellates |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Euglena | Trachelomonas | Chlamydomonas | Phacus | Total of four genera | Total of all flagellates | |
| 1928 | *Number* | | | | | | | | | |
| Impounded bayou | 15 | 0 | | 1,440 | 2,127 | 530 | 123 | 4,220 | 6,353 | 66.42 |
| Other nonbreeding | 10 | 0 | | 3,040 | 1,309 | 592 | 312 | 5,253 | 7,302 | 71.94 |
| Low larval density | [a]29 | 1–10 | 4.6 | 1,234 | 1,700 | 336 | 155 | 3,425 | 5,260 | 65.12 |
| High larval density | 22 | 11–35 | 19.7 | 1,793 | 1,026 | 1,207 | 60 | 4,086 | 5,446 | 75.03 |
| 1929 | | | | | | | | | | |
| Impounded bayou | 15 | 0 | | 1,320 | 935 | 1,348 | 167 | 3,770 | 6,882 | 54.78 |
| Other nonbreeding | 9 | 0 | | 5,097 | 1,328 | 1,281 | 447 | 8,153 | 11,028 | 73.93 |
| Low larval density | [a]17 | 1–10 | 5.3 | 522 | 588 | 922 | 102 | 2,134 | 2,824 | 75.57 |
| High larval density | 20 | 11–45 | 22.8 | 831 | 921 | 1,231 | 124 | 3,107 | 4,195 | 74.07 |

[a] 1 observation omitted, as in Table 2.

## EFFECT OF IMPOUNDING WATER ON ANOPHELES BREEDING

The fact that the waters of this locality are well stocked with the mosquito-destroying fish *Gambusia affinis* Raf. makes it necessary that protection of some sort be afforded Anopheles larvae before development can take place. This was well illustrated by Van Dine (7), who cleared and impounded a 1-mile section of one of the bayous near Mound and thereby eliminated the production of Anopheles in the area. (Fig. 2.) This section was completed in 1916, has been under observation continuously since that time, and has maintained itself mosquito free. Although other factors in addition to those of lack of vegetation and débris may be concerned in bringing about this condition in cleared and impounded areas generally, it is certain that unless protected from their enemies Anopheles larvae do not develop in numbers in this vicinity.

## DISCUSSION OF FACTORS IN LARVAL PROTECTION

Protection for larvae is afforded in waters in their natural state hereabout by floating vegetable débris, and by plants which grow on or at the surface of the water in such a manner as to conceal the larvae from their enemies. Floating débris may be more or less readily divided into two classes, viz, "large" débris and "small" débris. The former consists of logs, fallen trees, sticks, leaves, etc.; it does not form a particularly compact mass on the water surface and is not as effective in protecting the larvae as is the small débris. The latter is composed of small rotting particles of vegetable matter resembling very coarse sawdust which collect on the water surface and form mats of various sizes, in which the larvae are well protected from their enemies. Floating débris is much affected by wind and by the rising and falling water levels caused by alternating periods of rain and drought. In open areas and in the absence of vegetation or large débris to serve as anchorage, a wind will sweep the smaller material to shore, where a lowering water level will shortly strand it until a later rain causes the water to rise. In this manner good

larval protection may be alternately present and absent in the same location. This same process is in effect, of course, with the larger débris but to a lesser extent, as this material, especially the fallen trees and logs, tends to become lodged or anchored in place. Figure 3 shows an Anopheles breeding place formed by collections of débris.

Of the plants that serve as protection for Anopheles larvae, the most important perhaps are the filamentous algae which grow at or just below the surface film of the water. (Fig. 4.) Anopheles larvae usually thrive in their presence. Ceratophyllum, Potamogeton, Chara, and others of the larger plants which grow in the water and parts of which come to lie at the water surface, provide protection in essentially the same manner as the algae. Floating plants, such as Lemna, Heterantheria, Wolffia, Azolla, etc., can not be considered as effective protection for Anopheles larvae because of the fact that

FIGURE 3.—An uncleared bayou during high water, showing collections of Anopheles-sheltering débris

their leaves lie on top of the water surface and therefore do not hide the larvae from their enemies in the water. Patches of such plants, however, when not too dense, usually harbor attached algae or other materials which favor mosquito breeding. Furthermore, these algae and other materials tend to keep the leaves of the floating plants from forming a compact surface mat which would mechanically inhibit the production of Anopheles. In the same manner, plants such as Castalia, Nelumbo, Saururus, grasses, etc., which root on the bottom and extend to or through the water surface, while not of themselves providing much protection, serve as attachment and lodging for other protective materials.

Plants which have large root and stem masses below the water surface and send shoots above the water, sometimes in such profusion as to hide the water surface entirely, may or may not provide good

larval breeding areas. In this class are such plants as Jussiaea, knotweed, smartweed, climbing hempweed, etc. Jussiaea, for instance, by its habit of growth seems to offer excellent protection for larvae, and, when only small quantities of it are present in an area, larvae of all sizes are likely to be found among its algae-coated stems and roots. However, when an area becomes thickly covered with this plant, even though conditions in spots appear favorable, Anopheles breeding is often very sparse or wanting. While this plant grows luxuriantly under a variety of conditions, it shows a particular tendency to grow rapidly and to form a dense covering over the water surface in newly cleared or in cleared and impounded areas. For

FIGURE 4.—A patch of green algae mingled with débris. Anopheles larvae are usually abundant in such locations

some reason this luxuriant growth has not been observed to persist as a rule in the same location for several successive years.

## ADAPTABILITY OF LARVAE

Our common Anopheles mosquitoes are undoubtedly adaptable to a wide variety of breeding places, and, lacking their preferred habitat, they may readily choose another; that is to say, the fact that Anopheles larvae are not found in a certain location is no reason for believing that conditions in that area are such that they can not develop there. For example, in the course of airplane dusting operations a few years ago it was found that whereas the shrub and Nelumbo covered marginal areas in a large lake were breeding Anopheles, no larvae were present in a large central area of the lake which was more or less covered with growths of Castalia. Both areas had a thick subsurface growth of Ceratophyllum, Potamogeton, Utricularia,

algae, etc., which afforded excellent protection for larvae. A period of drought caused the marginal areas to become dry, and afterward intensive breeding occurred all over the Castalia-covered area.

## PLANTS AS CULICIDES

A summary and discussion of the literature on the larvicidal effect of plants on mosquitoes has recently been published by Matheson *(4)*. It is clear from his discussion that certain plants are definitely associated with lack of mosquito breeding in certain localities and not in others; in spite of considerable work on the subject, however, the exact factors causing these conditions are still unknown. It is impossible at the present time to ascribe to any of the plants growing hereabout definite larvicidal qualities against Anopheles mosquitoes, except those that are involved in limiting breeding in a purely mechanical manner, as will be shown later.

## EFFECT OF DÉBRIS AND OF ALGAE AND OTHER PLANTS ON LARVAL ABUNDANCE RATES AND PLANKTON CONTENT OF WATER

Table 4 shows the effect on the larval abundance rates of the presence and absence of débris in the breeding areas, irrespective of other protective agents, and also the effect of the presence of filamentous algae within each of these groups. It is here shown that in the areas lacking protective material no Anopheles breeding occurred and that in the areas containing large débris only the larval rate was much lower than in those where both large and small débris occurred. When filamentous algae are present with large débris the effect is to increase the larval rate considerably; when they are present with large and small débris, however, the larval rate is not essentially different than it is in their absence. These findings indicate that small débris and filamentous algae afford about the same degree of protection to Anopheles larvae. The percentage of large or mature larvae found in larval collections from the breeding areas is given to show that protection is afforded the insects by these conditions throughout larval life.

TABLE 4.—*Effect of débris and algae on larval abundance rates at Mound, La., 1928–29*

| Number of observations | Protective material | Larval rate per 10 dips | Large larvae |
|---|---|---|---|
| | | *Number* | *Per cent* |
| 7 | None | 0 | 0 |
| 48 | Large débris | 8.6 | 23.3 |
| 54 | Large and small débris | 13.01 | 23.7 |
| 21 | Large débris, no algae | 5.4 | 26.9 |
| 27 | Large débris, with algae | 11.2 | 21.7 |
| 14 | Large and small débris, no algae | 13.9 | 20.8 |
| 40 | Large and small débris, with algae | 12.8 | 24.6 |

Table 5 gives additional data on the occurrence of Anopheles larvae with protective materials and also shows the nature of the plankton content of the water in each of the groups of breeding areas. This table shows that the larval abundance rates in the groups having large débris alone, those having grass, weeds, etc., and those having

filamentous blue-green algae with and without weeds and grass are somewhat similar for both the years under consideration. In the groups with filamentous green algae alone, and those with a mixture of the green and blue-green material, the figures for the two years are not at all alike. In 1928 the presence of blue-green algae appears to have had a somewhat inhibitive effect on Anopheles breeding, as the stations having this material alone gave the lowest larval rate of any group in which filamentous algae were present, while those with green algae alone had a very high rate, and those with a mixture of the two forms had an abundance rate indeterminate between the two. In 1929, however, the effect of the presence of blue-green algae is not so clear, because, while the group of stations in which this material was found alone gave a lower rate than that in which green algae appeared alone, as in 1928, the group having a mixture of the two forms had the highest abundance rate. Large débris alone does not appear to afford very effective larval protection, but weeds, grass, etc., do. The presence of visible patches of filamentous green algae with large débris greatly increased the larval rate, either when found alone or when mixed with blue-green algae. When no algae other than blue-green was observed the larval density was much lower than that of any group in which algae occurred in association with débris.

TABLE 5.—*Effect of débris and other materials on Anopheles abundance rates and plankton content of the water, Mound, La.*

WITH LARGE DÉBRIS ONLY

| Year and observations | Larvae per 10-dip collection | Visible filamentous algae | | Grass, weeds, etc. | Organisms per cubic centimeter | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Green | Blue-green | | Total plankton | Flagellates | Ciliates | Amoeboids | Diatoms | Chlorophyceae | Cyanophyceae | Miscellaneous |
| 1928 | | | | | | | | | | | | |
| 9 | 2.6 | − | − | − | 5,624 | 4,072 | 417 | 50 | 555 | 269 | 11 | 250 |
| 6 | 10.2 | − | − | + | 5,829 | 3,917 | 325 | 4 | 1,425 | 154 | 4 | 0 |
| 2 | 4.0 | − | + | [b]± | 30,350 | 9,212 | 125 | 213 | 1,350 | 18,237 | 250 | 963 |
| 6 [a] | 7.8 | + | + | ± | 13,708 | 8,221 | 625 | 125 | 2,746 | 1,446 | 325 | 220 |
| 7 | 18.1 | + | − | ± | 8,400 | 4,125 | 354 | 18 | 2,357 | 1,189 | 193 | 164 |
| 1929 | | | | | | | | | | | | |
| 2 [a] | 0 | − | − | − | 18,225 | 12,688 | 350 | 0 | 150 | 37 | 5,000 | 0 |
| 3 | 8.0 | − | − | + | 8,808 | 7,350 | 225 | 8 | 717 | 467 | 8 | 33 |
| 2 | 6.0 | − | + | ± | 6,050 | 2,750 | 163 | 13 | 2,575 | 387 | 25 | 137 |
| 3 | 16.0 | + | + | ± | 4,625 | 825 | 100 | 67 | 275 | 1,867 | 1,483 | 8 |
| 6 | 9.9 | + | − | ± | 16,676 | 6,058 | 292 | 50 | 2,196 | 7,292 | 692 | 96 |

WITH LARGE AND SMALL DÉBRIS

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1928 | | | | | | | | | | | | |
| 5 | 14.2 | − | − | − | 6,260 | 4,640 | 200 | 5 | 940 | 455 | 20 | 0 |
| 3 | 10.3 | − | − | + | 12,925 | 11,225 | 675 | 33 | 492 | 467 | 33 | 0 |
| 0 | | − | + | ± | | | | | | | | |
| 3 | 17.5 | + | + | ± | 5,759 | 4,142 | 217 | 0 | 800 | 400 | 75 | 125 |
| 17 | 8.6 | + | − | ± | 7,223 | 5,546 | 377 | 26 | 677 | 418 | 48 | 131 |
| 1929 | | | | | | | | | | | | |
| 6 | 14.0 | − | − | − | 7,977 | 2,533 | 417 | 17 | 844 | 3,812 | 312 | 42 |
| 0 | | − | − | + | | | | | | | | |
| 2 | 2.0 | − | + | ± | 10,326 | 5,612 | 513 | 0 | 2,063 | 2,075 | 63 | 0 |
| 9 | 22.0 | + | + | ± | 4,707 | 2,872 | 117 | 3 | 928 | 364 | 355 | 68 |
| 9 | 13.1 | + | − | ± | 6,055 | 4,958 | 153 | 31 | 858 | 47 | 5 | 3 |

[a] 1 observation omitted.

[b] Plus-minus signs indicate that some of the dips of the 10-dip collection contained grass, weeds, etc., while in other dips this material was absent.

Table 5 also shows the effect of large and small débris, alone and in combination with other protective agents, on larval rates and on the plankton content of the water. When small débris is found in association with weeds and grass or with algae the rates of larval abundance are not markedly different from those in similar groups but with only large débris present. In those groups that combine large and small débris with the algae (as in the lower part of Table 5), tne group showing blue-green filamentous algae has a low larval rate. However, the group in which blue-green filamentous algae were found mixed with green filamentous algae had much higher rates than the groups that had green algae alone. This fact would seem to indicate that if there is any relation between the presence of filamentous blue-green algae and the scarcity of Anopheles larvae, this deterrent influence is overcome hereabout by the presence with it of green algae.

The data on plankton organisms show that each of the groups is well supplied with plankton food for the larvae, and that the groups having the highest larval rates tend to have the lowest plankton counts, as has been previously noted. It is not believed that this condition is particularly significant, except as it indicates that in this locality the most favorable breeding conditions occur in more or less shaded areas, while the chlorophyll-bearing organisms become more abundant where the water surface is open.

## EFFECT OF CYANOPHYCEAE ON LARVAL ABUNDANCE

Table 5 shows that filamentous blue-green algae, when unassociated with green filamentous algae in the breeding areas, apparently limits the production of Anopheles. This might readily be ascribed to the fact that the growth habit of the filamentous Cyanophyceae is such as to give very little protection to the larvae, since the patches of blue-green algae when found alone are usually small and are easily blown about by the wind, or when growing attached to débris they usually form only a very narrow fringe. However, Boyd (*2*) reported a negative relation existing between the unicellular Cyanophyceae and anopheline larvae, and recently Allison and Morris (*1*) have shown that blue-green algae possess the power of nitrogen fixation. In studying some factors in mosquito ecology, Senior-White (*6*) reached the conclusion that "saline ammonia is inhibitory to Anopheles breeding, save in the case of the *rossi* group, in amounts exceeding one part per million." It may be, therefore, that in the areas hereabout where large quantities of Cyanophyceae are present, the water is given a saline ammonium content sufficient to make it unfavorable for Anopheles production.

## EFFECT OF LEMNA ON LARVAL ABUNDANCE

The effect of the presence of *Lemna* spp. in various quantities on larval abundance and on the plankton content of the water is shown in Table 6. In this table the observations on the abundance of this material have been divided into four groups, viz, those without Lemna (0), those with only scattered patches (+), those with an abundance but not sufficient to give the water a continuous surface mat (++), and those with a complete surface mat of the plant (+++). It is noted that the presence of considerable quantities of this material

(Table 6 (+ +); fig. 5) usually indicates good breeding conditions for Anopheles, while a complete surface mat (Table 6 (+ + +); fig. 6) of Lemna effectively checks breeding. It would also appear from the records of 1928 that even small quantities of Lemna (Table 6 (+)) tend to increase larval rates. This condition was reversed in 1929, however, and it is believed that in general the quantity of Lemna present in locations of this type has no particular effect on larval protection and abundance. That larvae reach maturity in all these environments except that having a complete surface mat of Lemna is shown by the percentages of large, or approximately mature, larvae occurring in the collections.

TABLE 6.—*Effect of Lemna on larval rates and on plankton counts per cubic centimeter in Anopheles breeding areas at Mound, La.*

| Year and number of observations | Larvae in 10-dip collections | Large larvae | Quantity of Lemna | Organisms per cubic centimeter | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total plankton | Flagellates | Ciliates | Amoeboids | Diatoms | Chlorophyceae | Cyanophyceae | Miscellaneous |
| 1928 | | *Per cent* | | | | | | | | | |
| 36 | 8.17 | 29.3 | 0 | 23,970 | 6,867 | 434 | 17 | 1,437 | 1,684 | 13,425 | 106 |
| 17 | 11.35 | 28.5 | + | 7,362 | 4,683 | 386 | 95 | 1,086 | 768 | 67 | 277 |
| 6 | 15.67 | 30.8 | ++ | 7,958 | 3,546 | 629 | 21 | 2,541 | 946 | 75 | 200 |
| 3 | .33 | 0 | +++ | 1,875 | 1,175 | 108 | 0 | 325 | 200 | 0 | 67 |
| 1929 | | | | | | | | | | | |
| 25 | 12.72 | 21.7 | 0 | 9,030 | 4,950 | 235 | 4 | 801 | 2,304 | 721 | 15 |
| 18 | 7.22 | 17.7 | + | 31,977 | 5,777 | 240 | 42 | 1,725 | 24,066 | 76 | 51 |
| 4 | 25.75 | 6.8 | ++ | 4,550 | 1,244 | 87 | 50 | 469 | 1,381 | 1,175 | 144 |
| 0 | | | +++ | | | | | | | | |

Lemna in large quantities causes lack of activity among chlorophyll-bearing plankton by cutting off most of the sunlight from the water surface. This is illustrated by the figures in plankton density given in Table 6. As usual, the highest plankton counts are associated with the lowest larval rates except in the case of the group in which a complete mat of Lemna covers the water surface; here, on account of the absence of sunlight, the plankton count is low and, for mechanical reasons, Anopheles larvae are exceedingly scarce. If the observations on the occurrence of Anopheles larvae with Lemna are separated into groups on the basis of those made at stations having large débris in addition to Lemna and those made at stations having both large and small débris in addition to Lemna, it will be found that the same general conclusions with respect to abundance of Anopheles in the presence of increasing quantities of Lemna apply.

## HYDROGEN-ION CONCENTRATION OF WATER IN RELATION TO PRESENCE OF ANOPHELES LARVAE

At the time of each observation on larval abundance a hydrogen-ion determination of the water was made by the use of a LaMotte colorimetric set. Table 7 gives a record of these determinations, which are divided into three parts—those made in the impounded areas, those made in areas from which Anopheles was temporarily absent, and those made in areas in which Anopheles was found. The data show that the waters in this locality do not have a particularly wide pH range and that they are predominantly alkaline in reaction in each

FIGURE 5.—*Lemna* spp. in abundance on water surface; a good environment for Anopheles

FIGURE 6.—A small lake with the water surface entirely covered by *Lemna* spp. Anopheles larvae are exceedingly scarce or entirely absent in such places

group of stations. Most of the pH values in the impounded areas are slightly higher than in the other groups, the mean being 8.01, while the mean in the breeding areas is 7.42. Undoubtedly the higher pH value in the impounded areas is connected with the higher plankton content of these areas, as it is known that the photosynthetic action of the algae decreases the hydrogen-ion concentration of waters in which they occur. In Table 7 the readings have been divided, for each group of observations, into those made in the morning and those made in the afternoon. The afternoon readings in each group have a somewhat higher range than the morning readings, illustrating the diurnal variation in hydrogen-ion concentration discussed by Matheson and Hinman (*5*). The data give no indication that the hydrogen-ion concentration is essentially different in the breeding and nonbreeding waters. The five highest readings in the breeding areas were associated with high larval rates (from 14 to 37 per 10 dips). Except for these, there was little or no correlation between larval abundance and either high or low readings.

TABLE 7.—*Hydrogen-ion concentration of water areas at Mound, La., 1928 and 1929*

| Station | Time of day | Number of observations in which the pH readings came within the indicated ranges of hydrogen-ion concentration | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6.8–6.9 | 7.0 | 7.1–7.2 | 7.3–7.4 | 7.5–7.6 | 7.7–7.8 | 7.9–8.0 | 8.1–8.2 | 8.3–8.4 | 8.5–8.6 | 8.7–8.8 | 8.9–9.0 | 9.1–9.2 |
| Impounded bayous | Morning | 1 | ---- | 1 | ---- | 4 | 4 | 2 | 2 | 1 | ---- | ---- | 3 | ---- |
| | Afternoon | ---- | ---- | ---- | ---- | 1 | 2 | 4 | 3 | ---- | ---- | ---- | ---- | 2 |
| Other nonbreeding areas | Morning | 0 | 2 | 5 | 2 | 2 | 1 | ---- | ---- | 1 | ---- | 1 | ---- | ---- |
| | Afternoon | ---- | 1 | ---- | ---- | ---- | 1 | 3 | 1 | ---- | ---- | ---- | ---- | ---- |
| Breeding areas | Morning | 8 | 10 | 8 | 12 | 9 | 8 | 1 | 1 | ---- | ---- | 1 | ---- | ---- |
| | Afternoon | 3 | 0 | 6 | 9 | 7 | 4 | 1 | 1 | ---- | 1 | 1 | ---- | 2 |

## RESULTS OF LARVAL DISSECTION STUDIES

Dissections were made of 31 lots of larvae in 1928 and of 32 lots in 1929 (a total of 272 larvae) to ascertain the nature of the food ingested. The larvae were not killed at the time of collection but were placed in water from the collecting area, brought immediately to the laboratory and examined. In making these dissections a larva was placed in clear water, the head and the last two or three abdominal segments were removed, and then by the use of needles and forceps the gut was drawn out, placed in a drop of clear water on a slide, and the contents squeezed out. A cover glass was then applied and the mount examined. A summary of the results of these dissections is given in Table 8.

This table shows the number of times each genus of organism was found in the water from the collecting areas, the number of times the genus was represented in the gut contents of the larvae examined from these locations, and also the number of times the genus was found in the larvae when not observed in the water sample. It is seen from these records that most of the genera, whether commonly or rarely present in the breeding areas, are liable to ingestion by the larvae. Whether this is by choice or chance is problematical. The amount of maceration undergone by an organism in the mouth of a larva and its degree of digestibility undoubtedly influence greatly the

condition of the organism in the larval gut and consequently the chance of its being recognized. In watching, under the microscope, larvae in the act of feeding, no selection of food particles was observed, the larvae ingesting whatever was presented, provided it was of suitable size. The larvae feed upon filamentous algae in two ways. Sometimes they ingest the entire filament and sometimes they run a filament between the mandibles, chewing and sucking out the cell contents as the filament goes through the mouth, and then discard the empty filament. It is of course impossible to identify any material subjected to this latter method of treatment. When the entire filament is ingested, however, a sufficient number of cells remain intact to make identification possible.

TABLE 8.—*Organisms of Anopheles breeding areas, and those found in larval dissections, Mound, La.*

| Organism | Number of times encountered | | | Organism | Number of times encountered | | |
|---|---|---|---|---|---|---|---|
| | In water | In gut and in water | In gut only | | In water | In gut and in water | In gut only |
| Cyanophyceae: | | | | Chlorophyceae—Continued. | | | |
| Anabaena | 16 | 3 | 0 | Oedogonium | 29 | 15 | 12 |
| Lyngyba | 8 | 1 | 0 | Spirogyra | 26 | 8 | 0 |
| Nostoc | 7 | 2 | 0 | Tribonema | 10 | 1 | 1 |
| Oscillaria | 12 | 7 | 1 | Ulothrix | 6 | 1 | 1 |
| Chroococcus | 3 | 1 | 0 | Vaucheria | 5 | 1 | 0 |
| Merismopoedium | 3 | 0 | 1 | Others | 30 | 11 | 0 |
| Synechocystis | 0 | 0 | 1 | Diatoms: | | | |
| Others | 3 | 0 | 0 | Unicellular | 63 | 53 | 0 |
| Chlorophyceae: | | | | Filamentous | 11 | 4 | 1 |
| Actinastrum | 3 | 0 | 0 | Protozoa: | | | |
| Ankistrodesmus | 1 | 0 | 0 | Amoeboid— | | | |
| Arthrodesmus | 1 | 0 | 0 | Arcella | 19 | 10 | 20 |
| Characium | 2 | 1 | 1 | Difflugia | 8 | 1 | 3 |
| Chlorobotrys | 0 | 0 | 1 | Flagellata— | | | |
| Chlorococcus | 2 | 0 | 1 | Ceratium | 2 | 0 | 0 |
| Chlorella | 2 | 1 | 0 | Chilomonas | 14 | 1 | 0 |
| Closterium | 12 | 4 | 4 | Chlamydomonas | 45 | 26 | 1 |
| Coelastrum | 3 | 1 | 1 | Cryptomonas | 1 | 0 | 0 |
| Cosmarium | 5 | 3 | 2 | Dinobryon | 2 | 0 | 0 |
| Crucigenia | 9 | 2 | 1 | Euglena | 60 | 45 | 0 |
| Dictysphaerium | 1 | 1 | 0 | Eudorina | 14 | 1 | 1 |
| Euastrum | 1 | 1 | 0 | Glenodinium | 18 | 1 | 0 |
| Gleocystis | 2 | 0 | 0 | Gonium | 9 | 2 | 0 |
| Kirchneriella | 1 | 1 | 0 | Mallomonas | 1 | 0 | 0 |
| Ophiocytium | 12 | 0 | 0 | Notosolenus | 1 | 0 | 0 |
| Oocystis | 0 | 0 | 1 | Pandorina | 16 | 2 | 2 |
| Pediastrum | 12 | 2 | 0 | Phacus | 38 | 21 | 2 |
| Planktosphaera | 1 | 0 | 0 | Platydorina | 4 | 1 | 0 |
| Scenedesmus | 16 | 3 | 1 | Pleodorina | 2 | 1 | 0 |
| Staurastrum | 1 | 1 | 0 | Synura | 11 | 1 | 0 |
| Tetraedron | 16 | 0 | 1 | Trachelomonas | 62 | 39 | 0 |
| Tetraspora | 2 | 0 | 0 | Urceolopsis | 6 | 1 | 0 |
| Xanthidium | 1 | 0 | 0 | Uroglena | 1 | 1 | 0 |
| Cladophora | 2 | 0 | 0 | Others | 50 | 7 | 1 |
| Hormidium | 1 | 0 | 0 | Pollen | 7 | 4 | 5 |
| Mougeotia | 8 | 1 | 1 | Spores | 13 | 11 | 22 |

It is believed that the difference in the character of food materials rather than the exercise of any preference on the part of the larvae accounts for the fact that some available organisms are recognized in the gut in a larger percentage of cases than others which are apparently as readily available for food. Hard-shelled organisms such as diatoms and certain protozoans are not easily crushed and are nearly always found in gut examinations when they are present in the water, while soft-bodied ciliates and the rarer flagellates are seldom recog-

nized. Another factor to consider in discussing these results is that it is not definitely known just what organisms are most likely to be drawn into the current set up by the mouth parts of the larvae. Some organisms doubtless come within larval range more frequently than others because their favored stratum of existence is at or close to the surface film in which Anopheles larvae do most of their feeding. As the water sample from which plankton examinations were made included the top half inch of water, organisms are probably included which may usually be outside of the larval feeding range.

It is noticeable that certain organisms, notably amoeboid Protozoa, the green alga, Oedogonium, and spores, frequently appear in the gut when not found in the water examination. It is probable that these organisms were lacking in the water sample as a result of the method of sampling. In order to obtain water free of trash the samples were drawn from clear surface areas sometimes distant a few inches from the place where the larvae were feeding. Had the water examination included a more intensive study of the adherents of the protective materials these discrepancies might not have been so evident.

## SUMMARY

Observations were made on various environmental factors prevailing in Anopheles breeding and nonbreeding areas in order to determine if possible the factors which influence the increase or decrease of larval abundance.

Protection for larvae is necessary in the water areas studied on account of the presence in abundance of *Gambusia affinis*, a mosquito-destroying fish, and other natural enemies. Protection is provided in the breeding areas by floating vegetable débris and by various species of plants. The best protection is afforded by small floating débris and by filamentous algae. Water areas containing filamentous blue-green algae in the absence of filamentous green algae were not found to be very favorable for larvae. Lemna affords some protection to the larvae, but when it occurs in such profusion as to form a complete mat over the water surface it almost completely inhibits the breeding of Anopheles.

Larval abundance, as measured by the number of larvae occurring in collections of 10 dips of surface water, was not greatly affected by variations in the mean summer air temperatures, which in each month were above 70° F.

Under certain conditions a rapid decrease in surface water was found to reduce the number of larvae, as a result of the stranding of protective material.

The plankton content of the breeding and nonbreeding waters was computed on the basis of the occurrence of the various groups of organisms per cubic centimeter of surface water. It was found that as a rule larger numbers of plankton organisms occurred in the nonbreeding waters. This was explained in part by the fact that the breeding waters usually are more shaded and the surface is covered to a greater extent by vegetation and débris, and this condition results in the development of fewer chlorophyll-bearing organisms. The character of the plankton as regards composition by classes of organisms in the various groups of waters in which Anopheles breeds or does not breed was found to vary considerably, but no consistent variations

in composition between the two groups were found, nor were any consistent differences observed between the breeding waters having high and those having low larval rates. Data are given for the flagellate Protozoa, showing that the four commonest genera within this class compose approximately the same percentage of the total flagellates in each group of waters.

The range of hydrogen-ion concentration in the breeding and nonbreeding waters was found to be essentially the same, and all groups of waters were principally alkaline in reaction. The mean of the readings in an unshaded impounded bayou, however, was higher than that in the breeding areas (8.01 as against 7.42).

Examinations of the gut contents of larvae showed that all organisms of suitable size when present in the water are likely to be ingested, but that some available forms are present in the gut less often than others. This may have resulted from the fact that the preferred habitat of these organisms does not coincide with that in which larvae usually feed, or it may have been that the amount of maceration which the softer-bodied organisms undergo greatly reduced their chances of being recognized.

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# LIFE HISTORY OF THE RABBIT STOMACH WORM, OBELISCOIDES CUNICULI[1]

By JOSEPH E. ALICATA

*Junior Zoologist, Zoological Division, Bureau of Animal Industry, United States Department of Agriculture*

## INTRODUCTION

In 1923 Graybill (*8*)[2] described from domestic rabbits (*Oryctolagus cuniculus*) in the United States a new genus and species of stomach worm which he called *Obeliscus cuniculi*. The following year Graybill (*9*) noted that the generic name Obeliscus was preoccupied and proposed the name Obeliscoides, type *Obeliscoides cuniculi*, to replace Obeliscus Graybill, 1923. Morphologically *Obeliscoides cuniculi* is related to *Graphidium strigosum* (Dujardin, 1845), a stomach worm occurring in wild and domestic rabbits in Europe. In common with the latter, *Obeliscoides cuniculi* may be visibly injurious to its host. Schwartz and Shook (*18*) have noted that the European stomach worm of rabbits is known to produce disturbances of various sorts that affect the health of rabbits and that the American stomach worm of rabbits has been found to produce ulceration of the stomach wall.

Specimens of *Obeliscoides cuniculi* from domestic and wild rabbits have been received in the Zoological Division of the Bureau of Animal Industry from ten States, namely, Florida, Iowa, Kansas, Louisiana, Maryland, Nebraska, New York, Ohio, Texas, and West Virginia, and from the District of Columbia. It is evident, therefore, that this parasite is widely distributed in this country.

The morphological features of the adults of *Obeliscoides cuniculi* have been described by Graybill (*8*) and Chandler (*3*), but no information is given in their reports concerning the preparasitic development of these worms and of the immature stages within the host. Since information concerning the life history of a parasite is essential as a basis for rational control measures, it is important that the basic facts in the life history of *Obeliscoides cuniculi*, particularly those relating to its free-living stages, be ascertained. The investigation described in this paper was undertaken principally for the purpose of discovering facts in the preparasitic development of Obeliscoides that might lead to practical methods of controlling this parasite in rabbitries. This problem was suggested to the writer by Benjamin Schwartz, of the Zoological Division of the Bureau of Animal Industry, and was carried out under his direction and supervision.

## METHOD OF INVESTIGATION

Eggs of *Obeliscoides cuniculi*, obtained from several females, were transferred to small glass jars containing a mixture of fresh, sterile rabbit feces and animal charcoal. After the eggs had incubated for

[1] Received for publication Aug. 7, 1931; issued April, 1932.

[2] Reference is made by number (italic) to Literature Cited, p. 418.

about 10 days at room temperature (20° to 24° C.), the infective larvae were recovered by means of the Baermann apparatus. Preinfective larvae were recovered from these cultures by transferring a small quantity of the culture material to glass dishes containing water and isolating the larvae with the aid of a microscope.

In order to study the course of development of the preinfective larvae, Obeliscoides eggs were cultured in water. This method, however, was not suitable for the study of larval development, since the majority of the developing larvae disintegrated before reaching the infective stage. In one instance, however, the entire preparasitic development took place in a water culture.

## DESCRIPTION AND DEVELOPMENT OF THE EGGS

The eggs of *Obeliscoides cuniculi* are usually elliptical in shape and are provided with two thin membranes. In a series of measurements involving about 100 eggs the variation in length was from 75$\mu$ to 91$\mu$, and the variation in width was from 42$\mu$ to 53$\mu$. Graybill (*8*) states that the eggs are 76$\mu$ to 86$\mu$ long by 44$\mu$ to 45$\mu$ wide, whereas Chandler (*3*) reports a somewhat greater range in size, namely, 80$\mu$ to 92$\mu$ in length by 56$\mu$ to 64$\mu$ in width. Segmenting eggs as small as 60$\mu$ by 38$\mu$ and as large as 152$\mu$ by 45$\mu$ have been found occasionally, but such extreme sizes are rare and possibly such eggs are abnormalities.

FIGURE 1.—Egg of *Obeliscoides cuniculi* from fresh rabbit feces

Eggs found in fresh rabbit feces, which were examined a few minutes after they were passed, were in about the 32-cell stage, as shown in Figure 1. In tap water the eggs hatched in about 30 hours.

## DESCRIPTION AND DEVELOPMENT OF THE LARVAE

### PREINFECTIVE LARVAE

The first-stage larvae, shown in Figure 2, are characteristically rhabditiform and resemble the first-stage larvae of related strongyles. The newly hatched larvae are from 320$\mu$ to 330$\mu$ long; they increase in size gradually during the first stage. The principal measurements of these larvae, made at various times after hatching and while they were still in this stage, are as follows: Length, 375$\mu$ to 448$\mu$; maximum width, 18$\mu$; length of esophagus, 85$\mu$ to 115$\mu$; length of tail, 65$\mu$ to 83$\mu$. These measurements indicate that the first-stage larva grows considerably from the time that it has hatched until it is ready to molt.

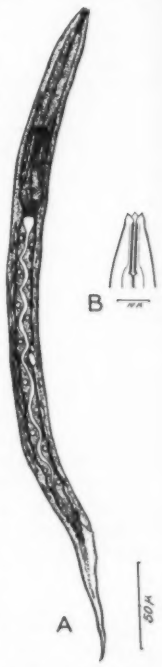


FIGURE 2.—A, First-stage larva of *Obeliscoides cuniculi*; B, anterior end of the first-stage larva showing lips and pharynx

The span of life of the first-stage larvae is comparatively brief; second-stage larvae were observed about 65 hours after hatching. The latter, as shown in Figure 3, A, differ but slightly from those of the previous stage. The outstanding difference is the larger size of the second-stage larvae. They range from 471$\mu$ to 750$\mu$ in length and

from 18$\mu$ to 22$\mu$ in maximum width, indicating considerable growth during this stage of development. During the second stage, as well as during the first, the larvae feed most of the time, and as development progresses the wall of the intestine becomes darker. Before the second lethargus is completed the opening of the pharynx closes, as shown in Figure 3, B.

FIGURE 3.—A, Second-stage larva of *Obeliscoides cuniculi*; B, anterior end of a late second-stage larva showing the mouth closed

INFECTIVE LARVAE

In 6-day-old cultures the larvae present were usually in the third stage; that is, they were in the ensheathed infective stage. In some cases, particularly when larvae had been kept in water for several days, the sheaths were absent because of their having been cast off.

Infective larvae appear less granular than preinfective larvae. (Fig. 4, A.) The former are from 653$\mu$ to 710$\mu$ long and about 22$\mu$ in maximum width. The maximum length of the infective larvae is somewhat less than that attained by the second-stage larvae. This is due to the fact that the tail of the infective larva is smaller than that of the second-stage larva. Several striking structural changes are apparent in this stage. The mouth is closed, and the constriction at the base of the lips has disappeared. The pharynx is no longer present, and there is a slight opening at the beginning of the esophagus. (Fig. 4, B.) The esophageal bulb has become more slender, and the valve is no longer present. The esophagus is from 167$\mu$ to 174$\mu$ long and is about one-twelfth the body length. The excretory pore is clearly visible and is located just below the nerve ring, approximately 110$\mu$ to 125$\mu$ from the anterior end of the body. The nerve ring appears as a light band just above the excretory pore, situated about

FIGURE 4.—A, Third-stage larva of *Obeliscoides cuniculi*; B, anterior end of the third-stage larva showing the absence of the pharynx

95μ to 102μ from the anterior end of the body, and encircling the esophagus obliquely. The genital primordium is located about 349μ to 400μ from the anterior extremity.

Table 1 shows the principal measurements of the first, second, and third stage larvae. These data show that the larvae grow considerably in the first stage and continue to grow in the second stage, at which time they attain a maximum length of 750μ, which is nearly two and one-half times the size of the newly hatched larvae. In the third stage the larvae neither grow nor develop beyond the stage which they had attained after the second molt.

TABLE 1.—*Principal measurements (microns) of 10 Obeliscoides cuniculi preparasitic larvae of each stage*

FIRST STAGE

| Items | Measurements of larva No. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Length of body | 375 | 375 | 382 | 383 | 395 | 402 | 418 | 440 | 440 | 448 |
| Maximum width of body | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Length of pharynx | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |
| Length of esophagus | 85 | 85 | 95 | 95 | 101 | 110 | 110 | 102 | 115 | 115 |
| Distance of nerve ring from the anterior extremity | 75 | 75 | 72 | 72 | 80 | 77 | 84 | 87 | 82 | 82 |
| Distance of excretory pore from the anterior extremity | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 110 | ---- |
| Distance between genital primordium and the anterior extremity | 203 | 203 | 205 | 205 | 223 | 215 | 213 | 216 | 218 | 228 |
| Length of tail | 65 | 65 | 71 | 68 | 76 | 76 | 83 | 80 | 79 | 83 |

SECOND STAGE

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Length of body | 471 | 524 | 570 | 577 | 585 | 560 | 615 | 669 | 722 | 750 |
| Maximum width of body | 18 | 18 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| Length of pharynx | 15 | 15 | 15 | 15 | 15 | 15 | 15 | 11 | 11 | 11 |
| Length of esophagus | 118 | 120 | 120 | 120 | 120 | 120 | 120 | 134 | 137 | 157 |
| Distance of nerve ring from the anterior extremity | 86 | 86 | 91 | 91 | 91 | 91 | 98 | 95 | 95 | 98 |
| Distance of excretory pore from the anterior extremity | 98 | 98 | 102 | 102 | ----- | 114 | 114 | 114 | 118 | ---- |
| Distance between genital primordium and the anterior extremity | 243 | 273 | 296 | 296 | 304 | 293 | 304 | 335 | 380 | ---- |
| Length of tail | 84 | 76 | 95 | 98 | 98 | 102 | 114 | 95 | 98 | 102 |

THIRD STAGE

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Length of body | 653 | 662 | 665 | 686 | 686 | 690 | 702 | 702 | 710 | 710 |
| Maximum width of body | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| Length of esophagus | 167 | 171 | 174 | 167 | 167 | 174 | 171 | 171 | 174 | 174 |
| Distance of nerve ring from the anterior extremity | 95 | 99 | 95 | 102 | 95 | 95 | 98 | 102 | 98 | 102 |
| Distance of excretory pore from the anterior extremity | 110 | 121 | 110 | 129 | 110 | 110 | 117 | 125 | 114 | 125 |
| Distance between genital primordium and the anterior extremity | 349 | 361 | 357 | 361 | ----- | 387 | 364 | 387 | 387 | 400 |
| Length of tail | 65 | 57 | 65 | 57 | 61 | 65 | 62 | 57 | 68 | 61 |

## EXPERIMENTS WITH INFECTIVE LARVAE

### ATTEMPTS TO INDUCE SKIN PENETRATION

A small drop of water containing about 100 larvae was placed on a portion of the skin of a young rabbit, from which the hair had been clipped. The rabbit was kept under restraint until the water evaporated. Two hours later a few drops of distilled water were placed on the area of skin that had been exposed to the larvae, and after

the water had remained on the skin for a short time it was transferred to a glass slide by means of a pipette. Practically all the larvae that were originally placed on the skin were recovered and were found to have retracted within their sheaths; after several minutes some of them became active. The area of the skin exposed to the larvae appeared normal. In another experiment, about 2,000 infective larvae were put on the skin of each of two rabbits, from which the hair had been clipped. One rabbit was killed eight days later and no larvae were recovered from the lungs and stomach. Examination of the lungs showed no petechial hemorrhages or other lesions indicative of infestation with nematode larvae. The second rabbit was killed 16 days after it had been exposed to a cutaneous infection; no worms were recovered from the lungs and stomach.

Another experiment was conducted in accordance with the technic described by Goodey (*4*). The skin of a 2-day-old rat was stretched, hair upward, on a cork ring, floated in a beaker containing warm physiological salt solution, and kept in an incubator at a temperature of 37° C. A small drop of water containing about 100 larvae was placed on the piece of skin and allowed to evaporate in the incubator. Two hours after the larvae had been placed on the skin and about 90 minutes after the drop containing larvae had evaporated, a drop of water was placed on the rat skin and then removed to a slide by means of a pipette. A microscopic examination revealed many larvae; they were still ensheathed. No larvae were found in the salt solution.

The rat skin was then fixed in 70 per cent alcohol, and the superficial layers were mechanically separated from the deeper layers. These layers were then cleared in an alcohol-phenol mixture. Several ensheathed larvae were found on the surface of the skin, but no larvae were found in the subcutaneous layers. This experiment was repeated by using the skin of a 3-day-old rat, and similar results were obtained.

These observations indicate that infection does not take place through the skin. Skin penetrators, such as the larvae of various species of hookworms, penetrate rat skin under the experimental conditions described above.

### REACTION TO COLD

Nematode larvae vary considerably in their ability to withstand low temperatures. Cameron (*2*) reports that infective larvae of *Monodontus trigonocephalus* did not revive after being frozen for a few minutes. According to Ransom (*15*), the infective larvae of *Haemonchus contortus* are very resistant to cold. He found that after larvae in sheep feces had been kept outdoors at temperatures ranging from 21.6° to −13.8° C. for 85 days they were still alive. Schwartz (*16*) reports that infective larvae of *Bustomum phlebotomum* (= *Monodontus phlebotomus*) which were frozen solid for about 15 hours, when thawed again became active. Schwartz and Price (*17*) found that the infective larvae of *Stephanurus dentatus* can withstand a temperature of −19° C. for six hours but are killed when exposed to this temperature for nine hours. Mönnig (*12*) found that the infective larvae of *Trichostrongylus* spp. from sheep were still alive after an exposure of 14 days to 0° C. Ortlepp (*13*) reported that the

infective larvae of *Triodontophorus tenuicollis* were able to withstand freezing when kept in an ice chest overnight. De Blieck and Baudet (*1*) pointed out that the infective larvae of the intestinal nematodes of the horse, *Strongylus vulgaris*, *S. edentatus*, and *Cylicostomum* spp., withstood a temperature of 0° C. for 15 days in cultures of water and feces. These writers also found that these larvae in feces survived after a 2-hour exposure to temperatures ranging from −15° to −20° C. When the larvae were placed in water and exposed again to the same temperature for six hours they remained alive. Raffensperger (*14*) exposed horse manure containing strongyle larvae of various species to Montana weather conditions for 20 months; some larvae did not succumb despite the fact that in the course of the experiment the temperature ranged from −18.3° to −38.° C. for a period of 26 days in January and February, 1929.

The effects of various low temperatures on the infective larvae of *Obeliscoides cuniculi* are summarized in Table 2. Each record is based on observations involving about 400 infective larvae. The larvae were put in glass tubes containing moist animal charcoal, and the tubes were placed in a refrigerator and removed from time to time for examination. Before the larvae were examined microscopically the tubes were kept at room temperature for about two hours.

TABLE 2.—*Effects of low temperatures on the infective larvae of Obeliscoides cuniculi, each culture involving about 400 larvae*

| Culture No. | Period of refrigeration | Temperature of refrigerator | Results |
|---|---|---|---|
| | *Hours* | *°C.* | |
| 1 | 5 | 0 | Larvae active. |
| 2 | 10 | 0 | Do. |
| 3 | 20 | 0 | Do. |
| 4 | 48 | 0 | Do. |
| 5 | 168 | 5 to 0 | Most larvae active, a few dead. |
| 6 | 168 | 0 to −4 | Do. |
| 7 | 360 | 0 to −4 | Do. |
| 8 | 720 | 2 to −4 | Do. |
| 9 | 24 | −18 | About 20 larvae active; all the others dead. The intestinal cells of all larvae vacuolated. |
| 10 | 72 | −18 | About a dozen larvae active; all the others dead. The intestinal cells of all the larvae vacuolated. |

In this experiment the Obeliscoides larvae were resistant to a temperature of from 2° to −4° C. for 720 hours. The temperature during this period ranged as follows: −4°, 240 hours; −3°, 24 hours; −2° to −1°, 24 hours; 0°, 384 hours; 1°, 24 hours; 2°, 24 hours. The vitality of most of the larvae kept at a temperature of −18° for three consecutive days was destroyed.

The resistance of the larvae to winter temperatures prevailing in the District of Columbia was tested as follows: About 1,000 or more infective larvae were placed in a jar containing sterile moist sand, and an equal number of larvae were placed in a jar containing sterile and slightly moist rabbit feces. These jars were kept outdoors for 30 days, from 11 a. m., December 15, 1930, to 3 p. m., January 14, 1931. The maximum temperature during this period was 13.9° C., and the minimum temperature was −9.4°. The total time during which the temperature was −1.1° or lower was 211 hours, so the

larvae were kept at a freezing temperature for about 29 per cent of the time during which they were exposed to outdoor conditions. At the end of this period the jars were removed to the laboratory and kept at room temperature for one day; a large number of active larvae were recovered from each jar by means of the Baermann apparatus.

### REACTION TO HEAT

Like the infective larvae of other strongyles, those of *Obeliscoides cuniculi* become very active when gradually warmed. Thus, if the end of a heated glass rod is brought in contact with the underside of a glass slide containing larvae, the larvae become active and orient themselves toward the source of heat.

The view expressed by Khalil (*10*) that only skin penetrators are positively thermotropic is untenable, as shown by observations recorded by various helminthologists. The larvae of *Monodontus trigonocephalus*, *M. phlebotomus*, and *Trichostrongylus* spp., as determined by Cameron (*2*), Schwartz (*16*), and Mönnig (*12*), respectively, are positively thermotropic, and the available evidence indicates that these larvae do not penetrate the skin of their hosts. The writer's observation concerning the heat reaction of *Obeliscoides cuniculi* lends additional support to the view that there is no necessary relation between the positive thermotropism of larvae and their ability to penetrate the skin of rabbits or other animals.

The effect of heat on the larvae of Obeliscoides was not considered to be of sufficient practical significance to warrant a study of their reaction to high temperatures.

### REACTION TO DRYING

The infective larvae of strongyles vary greatly in their ability to resist desiccation. Looss (*11*) reported that infective larvae of *Strongylus* spp. and *Cylicostomum* spp. can resist desiccation in a Petri dish for 14 days, and Raffensperger (*14*) noted that 10 per cent of *Strongylus* spp. larvae withstood desiccation in an incubator at 26° C. for a period of four months. Ransom (*15*) found that *Haemonchus contortus* larvae which had been dried in feces for 35 days revived after being moistened. Ortlepp (*13*) reported that infective larvae of *Triodontophorus tenuicollis* revived after they had been dried in an incubator overnight at 26° C. In contrast to these observations, Looss (*11*) has pointed out that larvae of *Ancylostoma duodenale* perish as soon as their surroundings become dry, and Goodey (*5*) has found that larvae of *Necator americanus* fail to revive after they have been dried a few minutes.

The following experiments were conducted to determine the resistance of Obeliscoides larvae to drying:

A small drop of water containing a number of infective larvae was placed on each of several glass slides. At the moment that the water appeared to have evaporated the time was noted, and the slides remained exposed at room temperature for various periods, as shown in Table 3. At the expiration of the desired lapse of time a few drops of water were added to the dried larvae and the preparations were examined at various intervals for about 24 hours. The results of these observations are given in Table 3.

TABLE 3.—*Summary of nine experiments on the resistance of infective Obeliscoides cuniculi larvae to air drying at room temperature* [a]

| Approximate number of larvae used | Length of exposure to drying | Condition of larvae after the addition of water |
|---|---|---|
| | *Minutes* | |
| 15 | 5 | All active. |
| 50 | 15 | Do. |
| 50 | 30 | 15 dead; all the others active. |
| 50 | 60 | 8 active; all the others dead. |
| 30 | 120 | 2 active; all the others dead. |
| 30 | 180 | 4 moved spasmodically; all the others dead. |
| 30 | 240 | 1 moved spasmodically; all the others dead. |
| 30 | 300 | All dead. |
| 30 | 360 | Do. |

[a] Approximately 23° C.

From Table 3 it is evident that the infective larvae of *Obeliscoides cuniculi* can withstand drying at room temperatures for several hours. Although some larvae succumbed after 30 minutes, others remained resistant for from 4 to 5 hours; however, the number of larvae that survived after 1 or more hours' exposure was relatively small. The survival period of *Obeliscoides cuniculi* infective larvae is longer than the survival periods of *Ancylostoma duodenale*, *Necator americanus*, *Monodontus phlebotomus*, and *M. trigonocephalus*.

### REACTION TO LIGHT

In a glass jar containing a 15-day-old culture the larvae were found crawling up the walls of the jar facing the light of a north window, but no larvae were present on the opposite side of the jar. This indicates that the larvae reacted positively to diffuse daylight. One-half of the surface of a Petri dish containing mature larvae in water, more or less evenly distributed in the dish, was covered with black paper, and the other half, facing a northern window, was left uncovered; 24 hours later most of the larvae had collected in the lighted half of the dish. This observation is in harmony with that described above. One-half of a Petri dish containing larvae in water was covered with black paper, and the half that remained uncovered was illuminated with a bright electric light which was placed about 14 cm. from the dish. Four hours later most of the larvae were found in the shaded portions. This indicates that the larvae are repelled by strong artificial light.

In their reaction to light the infective larvae of *Obeliscoides cuniculi* behave like those of *Trichostrongylus* spp. (*12*) and *Haemonchus contortus* (*19*). Cameron (*2*) reported that larvae of Monodontus are positively thermotropic and are not repelled by direct sunlight, or even by an electric light

### REACTION TO ANILINE STAIN

A drop of water containing about 25 larvae was put on a slide, and a cover slip was placed over it. A few drops of a 1 per cent aqueous solution of basic fuchsin were placed near one edge of the cover slip, so that the stain gradually filtered in and came in contact with the

larvae. The larvae remained active while in contact with the stain. They were kept under observation for an hour after the stain had run in under the cover slip, but no evidence of exsheathing was observed. The stain readily penetrated the sheath, but the tissues of the larvae remained unstained. The larvae continued active until the fluid evaporated. In one case larvae were kept in a vial with the stain for 18 hours, and at the end of that time all were active and sheathed.

In their failure to exsheath when in contact with a nonpoisonous stain, the infective larvae of *Obeliscoides cuniculi* resemble larvae of other nonskin-penetrating nematodes, such as *Monodontus phlebotomus* (*16*), *M. trigonocephalus* (*2*), *Trichostrongylus* spp. (*12*), *Hyostrongylus rubidus* (*6*), and *Uncinaria stenocephala* (=*Dochmoides stenocephala*) (*7*). The infective larvae of *Necator americanus* (*5*) and *Ancylostoma duodenale* (=*Agchylostoma duodenale*) (*11*) differ from the above-mentioned forms in that they exsheath when in contact with a solution of an aniline stain.

Cameron (*2, p. 212*), who worked with aniline stain, states that "all skin penetrators which have been tested have exsheathed, while the nonskin-penetrators have not exsheathed but have been quickly killed." Although there is still a possibility that all skin penetrators exsheath under the influence of the stain, it is evident that not all nonskin penetrators are quickly killed, as has been pointed out by Schwartz (*16*) and confirmed by the writer in experiments with larvae of *Obeliscoides cuniculi*.

With reference to the exsheathing process of the infective larvae of *Ancylostoma duodenale* in the presence of methyl green, Looss (*11*) states that "the experiment only succeeded beneath the cover glass, and even under these circumstances not unless a comparatively thin layer of fluid was allowed to remain between the cover glass and slide." Goodey (*5*) repeated this experiment with Necator larvae and obtained similar results. The writer was not able to confirm these observations in experiments with the infective larvae of *Ancylostoma caninum*. When a 1 per cent basic fuchsin solution was allowed to run in under a cover-slip preparation containing live larvae, the larvae were found to exsheath while a considerable quantity of fluid was still present between the slide and cover slip; when the larvae were immersed in a small vial containing a column of stain about 1.5 mm. high they exsheathed readily, despite the absence of pressure such as is afforded in a cover-slip preparation. The process of exsheathing in the latter case was observed under a binocular microscope.

## EXPERIMENTAL INFECTION OF RABBITS WITH OBELISCOIDES CUNICULI

In order to determine the length of time required for *Obeliscoides cuniculi* to develop to fertile maturity in its rabbit host, a number of feeding experiments were carried out. In these experiments ensheathed infective larvae were fed to noninfected domestic rabbits by mouth, essentially to determine the location of the worms and the character of the lesions. The results, which likewise include some worm counts and other supplemental data, are given in Table 4.

TABLE 4.—*Results of feeding infective Obeliscoides cuniculi larvae to domestic rabbits*

| Rabbit No. | Date of feeding larvae | Larvae | Date of appearance of eggs in feces | Days elapsing from time of feeding | Date of necropsy | Days after infection | Number and maturity of worms recovered | Location of worms | Lesions noted in stomach |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Sept. 26, 1929 | *Number* (a) | Oct. 16, 1929 | *Number* 20 | Oct. 26, 1929 | *Number* 30 | Numerous;[b] mature. | Several adult worms embedded in the stomach wall; the others free on the mucosa. | Petechial hemorrhages in mucosa. |
| 2 | Sept. 14, 1929 | (a) | (a) | (a) | Nov. 8, 1929 | 55 | do | As above; several worms completely covered by mucus. | Hemorrhagic areas. |
| 3 | Oct. 26, 1929 | (a) | (a) | (a) | Apr. 5, 1930 | 161 | 214 males; 118 females; mature. | Several worms embedded in the stomach wall; others free on the mucosa. | None. |
| 4 | do | (a) | (a) | (a) | Nov. 13, 1929 | 18 | Numerous;[b] mature and immature. | do | Congestion of mucosa and several large hemorrhagic areas. |
| 5 | Nov. 5, 1929 | 250 | Nov. 22, 1929 | 17 | Dec. 17, 1929 | 42 | 58 males; 39 females; mature. | do | Congestion of mucosa and numerous petechial hemorrhages. |
| 6 | do | 500 | Nov. 21, 1929 | 16 | Dec. 27, 1929 | 52 | 72 males; 92 females; mature. | do | Large masses of coagulated blood on stomach wall with numerous hemorrhagic areas. |
| 7 | do | 1,000 | do | 16 | do | 52 | Numerous;[b] mature. | do | Wall of stomach coated with a fine layer of coagulated blood; several scattered hemorrhagic areas. |
| 8 | do | 2,000 | Nov. 23, 1929 | 18 | Dec. 6, 1929 | 31 | 946; mature and immature. | do | Several petechial hemorrhages, with a few blood clots. |
| 9 | do | 2,700 | Nov. 25, 1929 | 20 | Nov. 26, 1929 | 21 | Numerous;[b] mature and immature. | do | Do. |
| 10 | Dec. 13, 1929 | 100 | Dec. 30, 1929 | 17 | Jan. 6, 1930 | 24 | 33 males; 22 females; mature. | do | Do. |
| 11 | do | 200 | do | 17 | Jan. 11, 1930 | 29 | 41 males; 22 females; mature. | Not recorded | Not recorded. |
| 12 | Dec. 16, 1930 | (a) | (a) | (a) | Dec. 22, 1930 | 6 | Numerous;[b] immature. | Several worms embedded in stomach wall; others free on the mucosa. | Congestion of mucosa with numerous petechial hemorrhages. |

[a] Undetermined.

[b] Not counted.

Table 4 shows that after experimental feeding of Obeliscoides larvae the minimum time required for the worms to reach egg-laying maturity was 16 days (rabbits 6 and 7) and the maximum time was 20 days (rabbits 1 and 9). However, the appearance of eggs in the feces does not indicate that all the females present had reached the egg-laying stage. Rabbits 4, 8, and 9, which were killed and examined post-mortem 18, 31, and 21 days, respectively, after they had been fed infective larvae, contained a number of immature Obeliscoides, although the feces of these rabbits had contained eggs before necropsy. Of the larvae fed, the percentage that reached maturity, as shown by the number of worms recovered at necropsy, ranged from 31 per cent in rabbit 11 to 55 per cent in rabbit 10.

Rabbit 3, which had been infested with Obeliscoides for a period of more than five months, had at time of its death about 400 eggs per gram of feces. On post-mortem examination 214 males and 118 females were found in the stomach; of these females, 7 were gravid and the others had degenerated ovaries and uteri (fig. 5), indicating that they had passed the stage of egg production and had become senile and sterile. The 7 gravid females yielded on an average 57 eggs per gram of feces for each egg-producing female. In contrast to this finding, rabbits 5 and 6, which harbored at necropsy only 39 and 92 adult females, respectively, a few days earlier had yielded 7,320 and 8,000 eggs per gram of feces, respectively. These observations show that the egg count is not necessarily a good index, in all cases, to the number of worms that an animal may harbor. The egg count gives no clue, for instance, to the presence of worms which are fully grown, but still agamic, nor to the presence of senile worms; neither does it account accurately for worm infestations in which there is a preponderance of males and for worm infestations in which, for one reason or another, there is a low egg production. By the use of the Stoll dilution technic it was found that rabbit 5 had 187 eggs per gram of feces for each of 39 female Obeliscoides harbored, 39 days after experimental infection and 3 days before the animal was killed. Rabbit 6, on the other hand, had only 87 eggs per gram of feces for each of 92 egg-producing females harbored, 50 days after experimental infection and 2 days before the animal was killed. This marked difference in egg production was

FIGURE 5.—Region of female reproductive organs of *Obeliscoides cuniculi*: A, Senescent female; B, gravid female

probably due to the difference in the age of the worms; the worms in rabbit 6 were probably past their prime in egg production.

The post-mortem examination of experimentally infected rabbits usually showed congestion of the gastric mucosa with numerous petechial hemorrhages. Erosion of the gastric glands and of the blood vessels was commonly observed in experimentally infected rabbits; large masses of coagulated blood also were found in the stomach

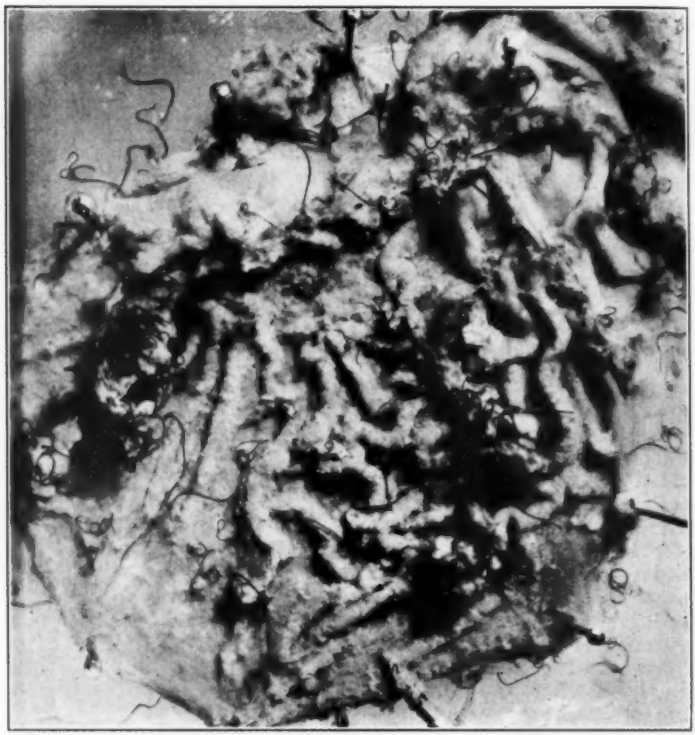

FIGURE 6.—Stomach of rabbit infested with *Obeliscoides cuniculi*. This rabbit was experimentally infected September 26, 1929, and examined post-mortem October 26, 1929

contents of such rabbits. The worms were usually free on the mucous membrane or deep in the stomach wall. Histological examination of infected areas of the stomach wall revealed worms under the mucous membrane; some worms were noted in the submucosa. In microscopic section, the gastric glands were found to be eroded. (Figs. 6, 7, and 8.)

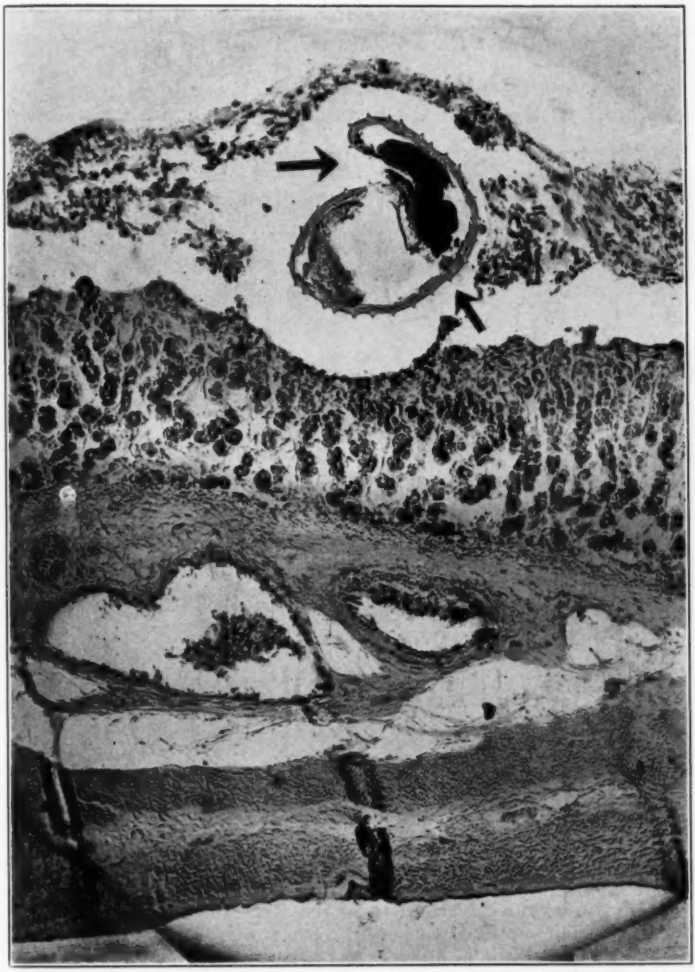

FIGURE 7.—Cross section of the stomach wall of a rabbit showing section of *Obeliscoides cuniculi* (indicated by arrows) on the mucosa. Note erosion of the gastric glands. This rabbit was experimentally infected September 14, 1929, and examined post-mortem November 8, 1929

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## EXPERIMENTAL INFECTION OF GUINEA PIGS WITH OBELISCOIDES CUNICULI

In order to determine the adaptability of Obeliscoides to a host other than the rabbit, a similar series of experiments was conducted which involved experimental infection of guinea pigs with infective larvae of this parasite. The results are given in Table 5.

Table 5 shows that Obeliscoides larvae can be successfully transmitted to guinea pigs. As far as can be determined from a survey of the literature, Obeliscoides has not been transmitted to guinea pigs heretofore. Of the larvae fed to guinea pigs, only a few succeeded in

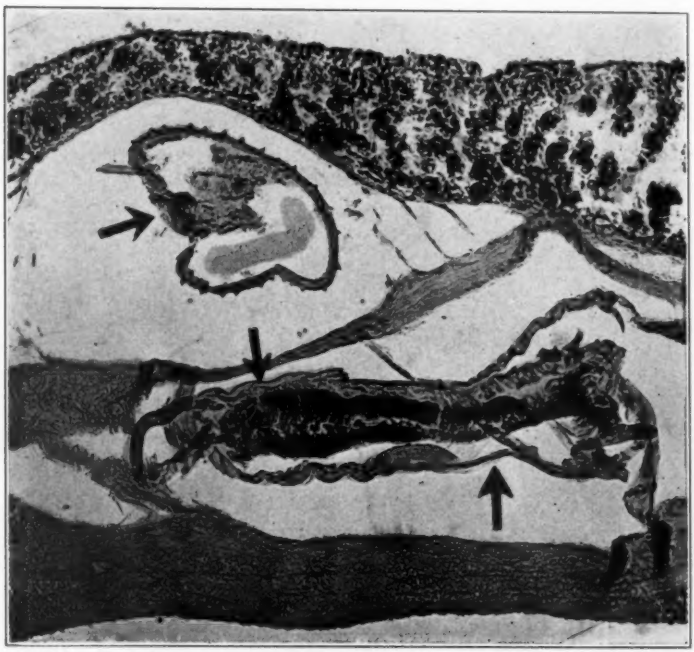

FIGURE 8.—Cross section of stomach wall of rabbit showing sections of *Obeliscoides cuniculi* (indicated by arrows) in the deeper part of the submucosa

reaching sexual maturity. In guinea pigs 3 and 4B, which were killed 2 and 7 days, respectively, after infection, more than one-fourth of the larvae that had been fed were recovered at necropsy; the worms thus recovered were still immature. From the findings on guinea pigs it may be assumed that the worms are eliminated from these animals to a large extent before reaching maturity. Guinea pig 7, which was fed about 200 larvae, contained 39 mature worms at necropsy, indicating that this particular animal was rather highly

susceptible to infestation with this nematode. The percentage of larvae that reached sexual maturity in guinea pigs was considerably lower than in most of the rabbits which were experimentally infected.

Lesions were observed in the stomachs of guinea pigs 3 and 7, in one of which a relatively large number of worms became mature. In guinea pigs 4B and 5, in which a relatively large number of immature worms were present, no stomach lesions were noted. In contrast to these observations, rabbit 12, in which the worms were also immature, had well-marked stomach lesions. Whether the rabbit is more susceptible to the injurious effects of Obeliscoides than the guinea pig remains to be determined.

TABLE 5.—*Results of feeding infective Obeliscoides cuniculi larvae to guinea pigs*

| Guinea pig No. | Date of feeding larvae | Larvae fed | Date of necropsy | Days after infection | Number and description of worms recovered | Lesions noted in stomach |
|---|---|---|---|---|---|---|
| | | *Number* | | *Number* | | |
| 1 | Dec. 16, 1929 | (a) | Jan. 11, 1930 | 26 | 1 gravid female | None. |
| 2 | do | (a) | Feb. 19, 1930 | 65 | 1 male | Do. |
| 3 | Jan. 20, 1930 | 200 | Jan. 22, 1930 | 2 | 58 third-stage larvae | Congestion of mucosa, with numerous petechial hemorrhages. |
| 4A | do | 200 | Jan. 25, 1930 | 5 | Number undetermined; immature. | None. |
| 4B | do | 200 | Jan. 27, 1930 | 7 | 75 fourth-stage larvae | Do. |
| 5 | do | 200 | Feb. 1, 1930 | 12 | 7 males and 16 immature females. | Do. |
| 6 | do | 200 | Feb. 13, 1930 | 24 | None | Do. |
| 7 | Jan. 27, 1930 | 200 | do | 17 | 17 males and 22 gravid females. | Congestion of mucosa, with scattered petechial hemorrhages. |
| 8 | Nov. 25, 1930 | (a) | Dec. 22, 1930 | 27 | None | None. |
| 9 | Oct. 17, 1930 | (a) | Nov. 7, 1930 | 21 | 1 male | Do. |
| 10 | do | (a) | Dec. 22, 1930 | 66 | None | Do. |

[a] Several hundred.

On several occasions the feces of infected guinea pigs containing Obeliscoides eggs were cultured by mixing the feces with animal charcoal and adding several drops of water to the mixture. The development of the eggs and larvae in such cases was normal.

### STAGES OF LARVAL DEVELOPMENT IN THE GUINEA PIG

In connection with the study of the susceptibility of guinea pigs to Obeliscoides, parasites in various stages of development were recovered from these animals after experimental infection.

Guinea pig 3, which was killed two days after experimental infection, yielded third-stage larvae; these worms showed the commencement of the third molt, as indicated in Figure 9. The principal measurements of six of these larvae were as follows: Total length, 856$\mu$ to 1,013$\mu$; maximum width, 26$\mu$ to 30$\mu$; length of esophagus, 186$\mu$ to 213$\mu$; distance of nerve ring from the anterior end, 114$\mu$ to 121$\mu$; distance of excretory pore from the anterior end, 133$\mu$ to 159$\mu$; length of tail, 57$\mu$ to 76$\mu$. The most significant morphological feature of these larvae, as shown in Figure 9, was the position of the genital

primordium, which had migrated posteriorly and was located approximately 170$\mu$ from the anus, in the particular specimen which was drawn. The position of the genital primordium in the various larvae recovered from this guinea pig varied, being from 80$\mu$ to 363$\mu$ from the anus.

FIGURE 9.—Third-stage larva of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig two days after experimental infection

Guinea pig 4A, which was killed 5 days after experimental infection, yielded fourth-stage larvae in which sex differentiation had become definitely established. The female reproductive apparatus showed considerable development. The vulva, ovejectors, uteri, and ovaries were definitely recognizable, as shown in Figure 10. At this stage of development the males may be distinguished by the inflated posterior end, which eventually forms the bursa.

The principal measurements of one female were as follows: Length, 2.26 mm; maximum width, 68$\mu$; length of esophagus, 357$\mu$; distance of nerve ring from the anterior extremity, 121$\mu$; distance of vulva from the tip of the tail, 440$\mu$; combined lengths of female reproductive apparatus, about 350$\mu$; length of tail, 87$\mu$.

Guinea pig 4B was killed seven days after experimental infection. This animal yielded fourth-stage females and males. The males were approaching the final molt, as evidenced by the sheath which is well separated from the body. (Fig. 11.) The bursa and its rays were still incompletely developed. The spicules were incompletely developed and only partly chitinized. The ejaculatory duct and the testis, the two being continuous, were fairly well developed. At this stage the ejaculatory duct is a slender tube opening into the cloaca and extending anteriorly for a short distance until it joins the testis. The latter is ventral to the intestinal tract. The females were less advanced in development than the males; they did not possess a sheath, a fact which indicates that they were not as yet approaching the final molt. The female genital apparatus at this stage is clearly differentiated into a pair of ovejectors sphincters, uteri, and ovaries. (Fig. 11.) The principal measurements of these worms were as follows: Males—length, 4.3 to 4.8 mm; maximum width, 114$\mu$ to 130$\mu$; length of esophagus, 483$\mu$ to 530$\mu$; distance of nerve ring from the anterior end, 182$\mu$ to 220$\mu$; distance of excretory pore from the anterior extremity, 273$\mu$ to 326$\mu$; length of spicule, approximately

FIGURE 10.—Fourth-stage larva of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig five days after experimental infection

320μ. Females—total length, 4.8 to 5 mm; maximum width, 98μ to 120μ; length of esophagus, 483μ to 530μ; distance of nerve ring from the anterior end, 197μ to 250μ; distance of excretory pore from the anterior extremity, 319μ to 349μ; distance of vulva from the anus, 748μ to 936μ; combined lengths of ovejectors 190μ to 230μ; length of tail 114μ to 121μ.

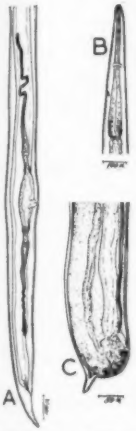


FIGURE 11.—Fourth-stage larvae of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig seven days after experimental infection: A, Posterior end of female; B, anterior end of female; C, posterior end of male

Guinea pig 5, killed 12 days after experimental infection, yielded fifth-stage worms or adults. (Fig. 12.) The males were unsheathed, a fact which indicates that the fourth molt had been completed and that the worms were in their final stage; the bursa and its rays resembled those of the adult forms, and the spicules were chitinized. The females, however, still retained the fourth or final sheath. The genital organs had developed considerably beyond the previous stage, the sphincters of the ovejectors and uteri were well developed and the ovaries were longer than in the previous stage and somewhat coiled. The ovaries were beginning to show developing ova; the posterior lobe of the ovary was looped, as shown in Figure 12.

The principal measurements of three males and three females were as follows: Males—length, 5.2 to 6 mm; maximum width, 125μ to 156μ; length of esophagus, 490μ to 639μ; distance of nerve ring from the anterior end, 234μ to 270μ; distance of excretory pore from the anterior end, 390μ to 421μ; width of spread-out bursa, 140μ to 171μ; length of spicules, 440μ to 452μ, corresponding with those of the adult forms. Females—length, 5.5 to 6.2 mm; maximum width, 202μ to 218μ; length of esophagus, 624μ to 655μ; distance of nerve ring from the anterior end, 265μ to 280μ; distance of excretory pore from the anterior end, 390μ to 436μ; distance of vulva from anus, 1,014μ to 1,138μ; combined lengths of ovejectors, 234μ to 265μ; length of tail, 109μ to 147μ.

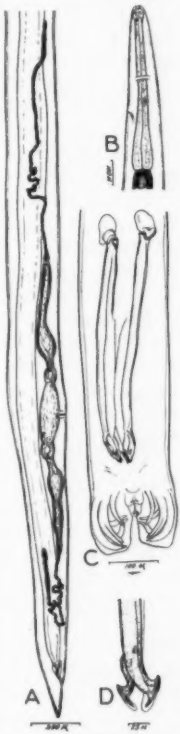


FIGURE 12.—Fifth-stage adults of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig 12 days after experimental infection. A, Posterior end of female; B, anterior end of female; C, posterior end of male; D, lateral view of tips of the right spicule

Guinea pig 7, which was killed 17 days after experimental infection, yielded adult worms of both sexes. Most of the females had segmenting eggs in the uterus, whereas the uteri of others contained no eggs.

The period required for Obeliscoides to reach egg-laying maturity in guinea pigs is about the same as that in rabbits. Development of Obeliscoides in both of these hosts, therefore, proceeds at about the same rate.

## SUMMARY

In common with those of other strongyles, the free-living larvae of *Obeliscoides cuniculi* undergo two molts in fairly rapid succession. In charcoal cultures and at room temperatures the infective stage is reached in about six days.

The infective larvae failed to produce skin lesions and subsequent infestations when placed on the intact skin of live rabbits. These larvae were incapable of burrowing into the skin of young rats under experimental conditions.

Infective larvae were found to withstand a temperature of 2° to −4° C. for 30 days, but the vitality of most of them was destroyed after being kept at a temperature of −18° C. for 3 days.

The infective larvae did not appear to be very resistant to desiccation, as all of them were found dead after a 5-hour exposure to air drying at room temperature.

The infective larvae responded positively to diffuse daylight but were repelled by strong artificial light.

In the presence of a 1 per cent solution of basic fuchsin, the infective larvae did not exsheath and remained active in the stain for a period of 18 hours.

Most of the infective larvae reached sexual maturity in the stomach of rabbits in 16 to 20 days.

Post-mortem examination of experimentally infected rabbits, within about a month after the larvae were fed by mouth, usually revealed areas of inflammation in the gastric mucosa and the presence of petechial hemorrhages and blood clots on the stomach wall. The worms were found free on the mucosa or embedded in the stomach wall.

The larvae undergo two molts in the stomach of the rabbit before attaining sexual maturity, the worms become sexually differentiated after the third molt, but the bursa is not fully developed until the fourth or final sheath has been cast off.

In several instances infective larvae were found to reach sexual maturity in the stomach of guinea pigs; in such cases the eggs developed normally and the larvae reached the infective stage after undergoing the usual two molts. The time required for Obeliscoides larvae to reach the egg-laying stage in the guinea pigs was about the same as in the rabbits.


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# SEASONAL SUBSOIL TEMPERATURE VARIATIONS[1]

By ALFRED SMITH

*Associate Soil Technologist, California Agricultural Experiment Station*

## INTRODUCTION

Actual diurnal fluctuations of temperatures follow the geometric progression law, and as found by Bouyoucos (*2, p. 131*)[2] "the diurnal-nocturnal amplitude of oscillation of temperature decreased in geometric progression as the depth increased in arithmetic progression, in all the different types of soil." Keen (*10, p. 308*), in discussing the theory of heat flow in a conducting material, emphasized the importance of the heat conductivity of the soil, which he defined "as numerically equal to the quantity of heat flowing per unit of time through unit area of a unit cube of material when unit temperature difference is maintained between two opposite faces." He also considered the specific heat, the apparent density, and the diffusivity of the soil, and assuming the application to the soil surface of a simple harmonic variation, showed how the amplitude of such a temperature wave diminished exponentially as the depth of observation increased.

Smith (*17, 18*) has reported that a distinct rise and fall in soil temperatures in a 24-hour period occurs to a depth of 12 inches, and the night temperatures for the 6-inch and 12-inch depths average higher than the day temperatures. The diurnal range in soil temperatures is influenced largely by the character of the sky, rainfall or soil moisture, and the direction and intensity of the wind (*17*). The highest soil temperatures in the surface soil as reported by Schucht (*15*) in Germany, are about 113° F., in the Russian steppes 140°, and in western Arizona 160°. Smith (*16*) reported that the highest temperature at Davis, Calif., in an area kept free from vegetation, and obtained at a depth of one-half inch, was 143°.

The soil temperatures that have usually been reported have been those occurring on the soil surface or at depths less than 3 feet. McClatchie (*12*) conducted soil-temperature investigations in the Salt River Valley of Arizona by the use of three self-registering thermometers situated underground at depths of 5, 10, and 15 feet. His results during the two years of investigation show that the annual range of temperature decreases with depth, for at 5 feet it varied from 20° to 25° F.; at 10 feet, from 15° to 20°; and at 15 feet, from 10° to 15°. He estimated that in the area under investigation, at a depth of about 50 feet, the soil temperature would remain constant throughout the year. Rambaut (*14*) determined the monthly temperatures in a gravelly soil under grass at Oxford, England, and found that the annual soil temperature range at a depth of 10 feet was 9.5°.

Callendar and McLeod (*4*) at Montreal reported soil temperatures from an area covered with a layer of turf, where the soil consisted of loose, light-brown sand to a depth of 8½ feet, below which to a depth

[1] Received for publication Sept. 9, 1931; issued April, 1932.

[2] Reference is made by number (italic) to Literature Cited, p. 427.

of about 30 feet there was a bed of stiff blue clay. Water was always found in the sand for a certain distance above the clay. The annual ranges in soil temperature were 34.3° F. at a depth of 20 inches, 26.0° at 40 inches, 19.5° at 66 inches, and 11.0° at 108 inches. The area in which these observations were taken was covered with snow from approximately January 1 to April 1.

Fitton and Brooks (*8*) have summarized the data which have been reported on soil temperatures in the United States up to 1931 in various soils under different conditions of cover and moisture at different elevations and exposures, and have made available in their discussion a very complete bibliography of literature on this subject.

## ROOT GROWTH AND SOIL TEMPERATURE

As the roots of certain plants, under favorable soil conditions, extend to depths greater than 3 feet, and during the summer months such roots in the deeper areas are in a soil climate cooler and in the winter warmer than their aboveground parts, it was deemed advisable to obtain soil temperatures to a depth of 12 feet.

The seasonal subsoil temperature changes even at depths of 12 feet are important, as a rise of temperature is brought about by the physical process of absorption, conduction, diffusion, and convection (*7*). A lowering of the temperature is brought about by a reciprocal process involving diffusion, conduction, vaporization, and radiation. The characteristics of the solid, liquid, and gaseous phases of the soil mass affect the rate of this movement.

The most favorable soil temperature for most crops, if other conditions are favorable, is usually considered as being between 65° and 70° F. (*19*.) Roots in the deeper soils grow vigorously at much lower temperatures, varying of course with the species (*20*). Cannon (*5*) found that the most rapid rate of root growth in seedlings of *Prosopis velutina* occurred at a soil temperature of about 93.2°, at which point roots with an initial length of 16 mm grew 51 mm in 12 hours. He found that not only was the rate of growth of shoots correlated with the temperature of the soil, but also with the length of the roots.

Under a diminished oxygen supply the effect of soil temperature seems to be greatly modified (*6*). As the oxygen supply in the soil is diminished, the rate of growth diminishes in a soil with a high temperature. In other words, crops in order to attain a fair rate of growth in time of high soil temperatures, must be in a well-aerated soil; otherwise, the rate of growth is considerably reduced. One of the most effective agents in soil ventilation is the changing daily or seasonal soil temperatures. The volume of a given mass of air is increased or diminished by $\frac{1}{491}$ of its original volume for each degree Fahrenheit change in temperature, the pressure remaining constant. Under such conditions a fall in temperature of 1° F. throughout a volume of 491 cubic feet of air would cause the entrance into the soil of 1 cubic foot of air. An increase in temperature of an equal amount would result in the movement outward of the same quantity of air (*11*). The effect of temperature upon soil aeration is not due, however, merely to the expansion and contraction of gases, but also to their differential absorption (*1*) by the soil at different temperatures. Alfalfa, red clover, field peas, and soybeans have been found to give a maximum nodule production at a soil temperature of about 75.2° (*9*).

## LOCAL CONDITIONS AND PROCEDURE

The area in which the soil temperatures were determined is located at Davis, Calif., on a recent alluvial fan which has a slope in the immediate vicinity of from 5 to 10 feet per mile. The texture of the soil in the various horizons is loam from the surface to a depth of 3 feet, fine sandy loam from 3 to 9 feet, coarse and fine sand from 9 to 11½ feet, and silt loam to 12 feet.

The rains in this district occur mainly between September and May, and as the area has been kept free from vegetation since 1923, and the water table has remained at about 20 feet, the soil is moist to field capacity during most of the winter season. During the dry season (May to September), as the area is not irrigated, the moisture content of the surface foot of soil is reduced by surface evaporation, but at greater depths the changes are slight. The moisture changes which usually occur in this area during the year have previously been described (*17*).

In November, 1928, electrical resistance thermometers were installed at depths of 4, 5, and 6 feet, and in September, 1929, at 8, 10, and 12 feet. The thermometer bulb consists of an insulated nickel winding inserted in a strong brass tube, serving as a case. The lead-covered leads are soldered with a water-proof joint to the stem of the thermometer and connected to a cable, which extends to a small building near the plots, where a resistance thermometer indicator of the balance type is located. In burying the thermometers, a hole of small diameter was dug, the soil of the various horizons being carefully laid aside. The thermometers were then inserted and the hole filled, the soil layers being put back in proper order and lightly tamped in order to attain the same degree of compactness as existed originally. The soil temperatures at these depths change very slowly and weekly readings were therefore sufficient.

## INTERPRETATION OF TEMPERATURE DATA

Subsoil temperatures at the same depth vary from year to year. During certain parts of the year the subsoil temperatures may remain fairly constant for several weeks, and for this reason 4-week averages were determined for various depths ranging from 4 to 12 feet, inclusive. These averages are shown in Figure 1. In Figure 1, particular attention is called to the data obtained in 1930, when during November the temperatures did not vary appreciably in the 4 to 12 foot depths. It appears that May and November are the pivotal months of the year. The time of occurrence of the minimum and maximum average soil temperatures as related to depth are clearly illustrated.

Taking the weekly readings, it was found that the temperature ranges in 1930 at the various depths, as shown in the lower right-hand corner of Figure 1, were 27° F. at 4 feet, 22° at 5, 18° at 6, 14° at 8, 12° at 10, and 9° at 12 feet. With increasing depth the amplitude of temperature changes per foot of depth were of lesser magnitude in the deeper subsoil than in the upper subsoil.

Air temperatures were obtained in a standard United States Weather Bureau shelter at Davis, Calif., near the area where the subsoil temperatures were determined. The lowest air temperature in 1929, which was 23° F., was recorded on February 11. The mean

monthly temperature for January of that year was 40.4°, and for February 46.9°. The highest air temperature in 1929 was 111°, on June 25. The lowest air temperature during the first part of 1930 was 24°, on January 13, and the highest during the year was 107°, on July 14. The time of occurrence of the minimum and maximum soil temperatures in 1930, at depths ranging from 1 foot to 12 feet, is practically a straightline function of the depth. (Fig. 2.) The minimum at the 1-foot depth occurred on the same day as the minimum air temperature, while at a depth of 12 feet the minimum did not occur until May 5, or 16 weeks later. In like manner, the maximum temperature at the 1-foot depth in 1930 occurred shortly after the maximum air temperature, while at a depth of 12 feet the maximum was not reached until November 3, or 15 weeks later.

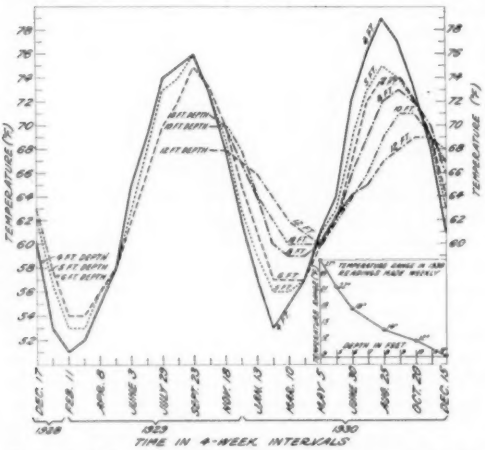


FIGURE 1.—Subsoil temperatures by 4-week averages at depths varying from 4 to 12 feet, Davis, Calif., December 17, 1928, to December 15, 1930

The time of the occurrence of the minimum and maximum soil temperatures as compared to the minimum and maximum air temperatures, or the lag, is not the same each year. During some years this lag at the 3-foot depth may be 80 hours (*17*), while in 1930 it ranged from 2 to 3 weeks. This is due to the varying moisture content of the soil and is also affected by the character of the air temperature preceding and following the time of occurrence of the minimum and maximum temperature. In other words, during the week preceding or following the occurrence of the maximum air temperature, the air temperatures may have been as much as 10 degrees lower or perhaps within 2 degrees of the maximum.

The changing soil temperatures throughout the year can also be illustrated by determining the mean monthly temperatures (fig. 3), and in this way the ebb and flow of heat can be better understood. As previously stated, the soil temperatures at depths of 4 to 12 feet, inclusive, were obtained by weekly readings.

During most of 1930 continuous temperature records were also obtained for various depths less than 4 feet by means of electrical resistance thermometers and an automatic temperature recorder.

From data obtained during previous years and by the use of mean monthly air temperatures, the average monthly soil temperatures were determined for those months in 1930 when the automatic temperature recorder was not operated. The data, therefore, for depths less than

4 feet for some months—May, October, November, and December—were in part interpolated. In January the upper subsoil has the lowest temperature and the soil is progressively warmer in the lower depths. In the month following, the upper subsoil becomes warmer while the deeper subsoil becomes cooler. The lowest average monthly temperatures for the lower subsoil (12 feet) is in April, while at a depth of 1 foot the average April temperature is 60° F. as compared to 47° in January. It will be noticed further that the average April temperatures are nearly the same (60°) for all depths from 6 inches to 12 feet.

The upper subsoil continues to become warmer until July, and then becomes cooler in August, while the deeper subsoil is warmer in August than in July. In fact, the highest average monthly temperatures at 10 feet occur in October, and at 12 feet the average temperatures for October and November are practically the same. The upper subsoil cools rapidly from September to January. Keen (*10*) in discussing Rambaut's results has pointed out that the periodic nature of the temperature wave closely approaches a 6-monthly symmetry.

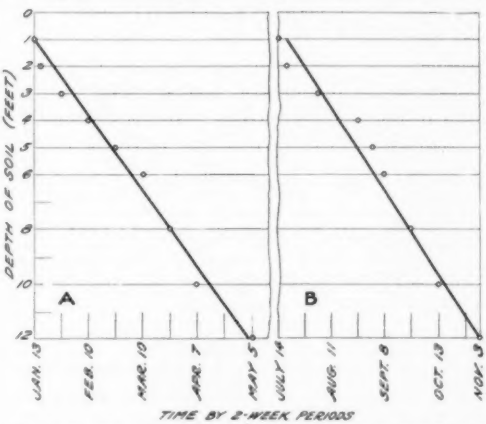


FIGURE 2.—Occurrence of minimu (A) and maximum (B) soil temperatures at depths varying from 1 to 12 feet, Davis, Calif., 1930

The subsoil temperatures, particularly at depths of 4 feet or more, show more accurately the total amount of heat absorbed by the soil than the temperatures at depths less than 4 feet, as the latter are more greatly affected by fluctuations in air temperatures. The heat conductivity into the soil is also illustrated from 10-year averages obtained in bluegrass sod in Illinois (*13*) where the highest average monthly temperature (73.5° F.) at a depth of 9 inches was reached in July, while at a depth of 36 inches the highest average monthly temperature (67.5°) was reached in August.

In Table 1 the monthly mean air temperatures and the rainfall for 1930 and the departures from the normal are shown. The normal monthly mean temperatures and rainfall are based on records extending for 23 to 59 years, respectively. The monthly mean temperatures reported (*3*) were determined from maximum and minimum thermometers exposed in a standard United States Weather Bureau shelter 4½ feet above the surface of the soil, a short distance from the soil-temperature plots.

TABLE 1.—*Monthly mean air temperatures and rainfall, with departures from the normal, at Davis, Calif., 1930*

| Month | Monthly mean air temperature | Departure from normal | Monthly rainfall | Departure from normal |
|---|---|---|---|---|
| | ° *F.* | ° *F.* | *Inches* | *Inches* |
| January | 43.6 | −1.0 | 3.80 | −0.07 |
| February | 52.0 | +3.4 | 1.66 | −1.15 |
| March | 54.0 | +1.1 | 3.48 | +1.07 |
| April | 57.2 | 0 | .92 | −.17 |
| May | 59.0 | −4.1 | .18 | −.45 |
| June | 70.6 | +.6 | 0 | −.14 |
| July | 72.7 | −1.9 | 0 | −.01 |
| August | 73.0 | −.2 | 0 | −.01 |
| September | 65.6 | −3.6 | .23 | −.10 |
| October | 59.3 | −2.1 | .69 | −.03 |
| November | 53.6 | +1.8 | .92 | −.69 |
| December | 44.6 | 0 | .20 | −3.20 |
| Annual mean | 58.8 | −.5 | 12.08 | −4.95 |

The monthly mean air temperatures in 1930 for February, March, June, and November were higher than the normal, and during the remaining months of the year they were either equal to, or fell below, normal. In February, 1930, the mean monthly air temperature was 3.4° F. higher and in May it was 4.1° lower than the normal. It was during these two months that the greatest departures in the air temperatures from the normal occurred.

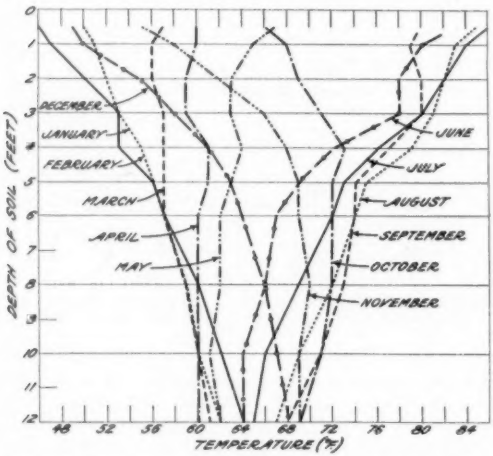


FIGURE 3.—Monthly averages of soil temperatures in degrees Fahrenheit at depths varying from 1 to 12 feet, Davis, Calif., during 1930

The rainfall was above normal in one month only (March), when the departure amounted to 1.07 inches. The greatest departure in rainfall from the normal occurred in December of 1930, when the precipitation was 3.20 inches below normal.

The annual average soil temperatures for depths varying from 6 inches to 12 feet for the year 1930 were found to range from 65 to 67° F. At 6 inches, 1 foot, and 12 feet the average was 65°, at 3 feet and 10 feet it was 67°., and for the other depths it was 66°. The average annual mean air temperature, based on records obtained from minimum and maximum thermometers, is 59.3°, and during 1930 it was 58.8°. The annual average soil temperatures for 1930, at all depths from 6 inches to 12 feet, were therefore higher than the mean air temperature for 1930 determined from minimum and maximum thermometers.

## SUMMARY

Soil temperatures were determined by means of electrical resistance thermometers at Davis, Calif., in an unirrigated area that was kept free from vegetation during the experiment and for 6 years before the experiment. The water table stood at a depth of 20 feet. During the wet season, September to May, the soil was generally moist to field capacity. During the dry season the greatest loss of moisture occurred from the surface foot of soil.

During 1930, May and November appeared to be pivotal months with relatively uniform soil temperatures at all depths from 4 to 12 feet. The annual temperature ranges for the various depths were 27° F. at 4 feet, 22° at 5 feet, 18° at 6 feet, 14° at 8 feet, 12° at 10 feet, and 9° at 12 feet. In the lower subsoil the temperature ranges per foot increase in depth were not of such great magnitude as in the upper subsoil.

The time of occurrence of the minimum and maximum soil temperatures at depths ranging from 1 foot to 12 feet is practically a straight-line function of the depth. At the 1-foot depth they occurred on the same day as the minimum and maximum air temperatures, while at a depth of 12 feet the minimum soil temperature did not occur until 16 weeks later, and the maximum not until 15 weeks later.

The average monthly soil temperatures for depths ranging from 6 inches to 12 feet show that during the early part of the year the upper subsoil has the lowest temperature and the subsoil is progressively warmer with the depth. Although the upper subsoil becomes progressively warmer in February, March, and April the lower subsoil becomes progressively colder, attaining in April its lowest average monthly temperature for the year. In April the average temperature (60° F.) is nearly the same for all depths from 1 foot to 12. The upper subsoil continues to become warmer until July, and then becomes cooler in August, while the deeper subsoil is warmer in August than in July. At a depth of 12 feet, the highest average temperatures occur in October and November. A 6-monthly symmetry is shown by the temperature waves.

The annual average soil temperatures for depths ranging from 6 inches to 12 feet were found to vary from 65° to 67° F., while the annual mean air temperature, based on records obtained from minimum and maximum thermometers, was 58.8°


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# A METHOD FOR THE DETERMINATION OF COMPARATIVE HARDINESS IN SEEDLING ALFALFAS BY CONTROLLED HARDENING AND ARTIFICIAL FREEZING [1]

By GEORGE L. PELTIER, *Plant Pathologist, Nebraska Agricultural Experiment Station*, and H. M. TYSDAL, *Associate Agronomist, Division of Forage Crops and Diseases, United States Department of Agriculture*

## INTRODUCTION

In a recent publication the writers (*5*) [2] pointed out that 2-year-old, field-grown alfalfa plants were not found to be satisfactory for use in comparative controlled-hardiness tests. The variability of both the environmental factors and the plants within any one sort was so great that comparisons between different alfalfas varying in hardiness within small limits could not be made. Striking differences, however, were obtained between hardy, midhardy, and nonhardy alfalfas. In order to decrease this variability materially and shorten the time element, seedling alfalfa plants, grown entirely under controlled conditions in the greenhouse, were studied to determine whether or not they can be employed in comparative hardiness tests.

The primary object of this investigation was to develop adequate methods under controlled conditions that would give (1) consistent hardiness values comparable to field results; (2) a suitable procedure for the selection of hardy plant types; and (3) standard conditions that would serve as a basis for fundamental studies concerning the nature of hardiness in alfalfa and other crop plants.

## EQUIPMENT

The studies herein reported were carried out with the controlled hardening and freezing equipment recently described by Peltier (*3*). The hardening chamber was maintained at a uniform temperature slightly above 0° C. The temperatures in the freezer room were varied, depending on the temperature and length of exposure desired. The temperature in the freezer and the temperature of the materials under test were determined with copper-constantan thermocouples by means of a potentiometer and galvanometer. In later experiments a 16-point Leeds and Northrup resistance thermometer recorder was substituted for the thermocouples.

## METHODS OF PLANTING AND GROWING THE SEEDLINGS

### GENERAL METHODS AND PROCEDURE

It was found in preliminary trials that hard seeds sometimes caused confusion in the survival counts, especially when young seedlings were tested, because these seeds germinated after the artificial freezing. To avoid this complication small quantities of all alfalfa seeds, as needed, were treated for 10 minutes with concentrated sulphuric acid

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[2] Reference is made by number (italic) to Literature Cited, p. 444.

and immediately thereafter thoroughly washed with tap water and spread to dry.

Enough soil was prepared at the beginning of each year for the entire season. This consisted of finely shredded sod soil to which some sand was added. This mixture was uniform in texture and high in fertility, so that it was possible to adjust soil moisture readily and grow healthy and vigorous plants.

Several methods of seeding were followed, depending on the type of container and the nature of the experiment. As a rule, all sowings were heavier than necessary, and the plants were later thinned. In the small containers the seed was sown broadcast. If there were fewer than eight plants in a container it was discarded. The small containers usually had from 10 to 20 plants, the average being about 15. Apparently the difference in the number of plants per container within these limits was not important, as no decrease in variability occurred when the plants in the pots were thinned to a uniform number.

Two methods were used in sowing the seeds in the large containers: (1) Sowing broadcast a different alfalfa in each of the four quarters of the flats, and (2) sowing the seed in rows lengthwise of the flats and thinning ordinarily to 20 to 30 plants per row. Precautions were taken not to sow the seeds too near the outer edges of the flats.

The writers have been unable to find any publications dealing with comparative hardiness tests of young alfalfa seedlings under controlled conditions. For the most part previous investigators (*5*) have employed plants grown either in the greenhouse or field at least six months or longer, and in no instance were the plants subjected to controlled hardening. In this study all seedlings were grown under optimum conditions in the greenhouse in several types of containers usually for about one month, but occasionally for two. The plants were then placed in a hardening chamber for periods of 2 to 39 days and subsequently exposed to low controlled temperatures, so that in general the age of the plants at the time of the artificial freezing tests did not exceed 8 weeks.

In all tests the plants were counted just before they were transferred to the hardening chamber and again after they had recovered from freezing. Thus, a percentage of survival based on the total number of plants was obtained. A system of determining relative survival or injury by observation, as employed by Hill and Salmon (*1*), was not adopted because actual counts were considered more accurate. No notes on injury to the tops were taken, for generally the tops were completely killed back to the crown, from which the new growth usually started.

A preliminary series of tests was carried out to determine when survival counts should be made after the plants were artificially frozen. The new growth was observed in some instances in less than one week after freezing. It was found that at the end of two weeks all plants that had survived the artificial freezing recovered and produced sufficient new growth to be readily counted. Subsequent counts made at periods up to four weeks after freezing did not materially change the final number, although usually a slightly lower survival was found at the end of four weeks because a few plants died after initial recovery. The average of 36 readings showed a 40 per cent survival two weeks after freezing and a 38 per cent survival four weeks after freezing. The results of these tests show that reliable

counts of the plants can be made two weeks after freezing. All final counts in the cold-resistance tests were made two weeks after the plants were exposed to low controlled temperatures.

### INFLUENCE OF THE TYPE OF CONTAINER ON THE VARIABILITY OF THE PERCENTAGE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

The following four types of containers were employed in these studies: (1) Cypress flats (15 by 18 by 6 inches); (2) metal water-tight flats of the same dimensions; (3) porous clay pots (4 inches in diameter); and (4) square metal water-tight cans (4 by 4 by 6 inches). The plants were grown in these several types of containers to determine the type most suitable for comparative cold-resistance tests, particularly from the standpoint of least variability in percentage survival of the seedlings after artficial freezing. The plants used in these experiments were uniformly 1 month old and had been hardened for 15 days before freezing.

The standard deviation of a single determination, the coefficient of variability, and Weinberg's coefficient of variability of the percentage of survival of the seedlings after freezing are given in Table 1 for the different types of containers. The standard deviation of a single determination of all the tests which were extremely low or extremely high in percentage survival of the seedlings is less than that of the tests with intermediate percentages of survival. The average standard deviation for all containers with a survival from 76 to 100 per cent is 9.1, and from 0 to 24 per cent is 10.6, whereas the standard deviation for all containers with a survival from 25 to 75 per cent is 24.8. This is to be expected, since a very low or very high percentage of survival of the seedlings tends to eliminate actual variations because the freezing temperatures may be so high that none of the seedlings are killed or they may be so low that all the seedlings are killed. In either case there would be no variability.

TABLE 1.—*The influence of the type of container on the variability of the percentage survival alfalfa seedlings after exposure to low controlled temperatures*

PLANTS IN CYPRESS FLATS

| Kind of alfalfa | Average survival | Replications [a] | Standard deviation of a single determination | Coefficient [b] of variability | Weinberg's [c] coefficient of variability |
|---|---|---|---|---|---|
| | *Per cent* | *Number* | | | |
| Turkestan | 77.0 | 24 | 20 | 26 | 4.2 |
| | 47.0 | 8 | 24 | 51 | 5.6 |
| | 41.0 | 8 | 29 | 71 | 6.2 |
| | 32.0 | 8 | 19 | 59 | 5.0 |
| Nebraska common | 29.0 | 8 | 20 | 69 | 5.2 |
| | 28.0 | 8 | 20 | 71 | 4.9 |
| | 24.0 | 8 | 21 | 88 | 5.6 |
| Arizona common | 5.0 | 8 | 7 | 140 | 3.6 |
| | 2.0 | 8 | 4 | 200 | 3.0 |
| | .6 | 8 | 1 | 167 | 1.2 |

[a] 15 to 30 plants per replication in the large containers and 10 to 20 plants per replication in the small containers; month-old plants hardened uniformly for 15 days at 2° to 3° C. previous to freezing; length of exposure and temperature varied with each test, depending on the type of container.

[b] C. V. $= \frac{\sigma}{M} \times 100$.

[c] $W = \frac{\sigma \sqrt{Mn\text{-}Mo}}{\sqrt{(Ma\text{-}Mo)\ (Mn\text{-}Ma)}}$ where M = mean, Mn = the highest value, Mo = the lowest value, Ma = mean of all variants.

TABLE 1.—*The influence of the type of container on the variability of the percentage survival of alfalfa seedlings after exposure to low controlled temperatures*—Continued

| Kind of alfalfa | Average survival | Replications [a] | Standard deviation of a single determination | Coefficient [b] of variability | Weinberg's [c] coefficient of variability |
|---|---|---|---|---|---|
| | | PLANTS IN METAL FLATS (CONSTANT MOISTURE) | | | |
| | *Per cent* | *Number* | | | |
| Turkestan | 37 | 24 | 16.1 | 44 | 3.9 |
| | | PLANTS IN METAL CANS (CONSTANT MOISTURE) | | | |
| Turkestan | 95.0 | 6 | 3 | 3 | 2.1 |
| | 87.0 | 6 | 4 | 4 | 2.3 |
| | 86.0 | 7 | 8 | 9 | 4.4 |
| | 75.0 | 7 | 8 | 11 | 3.4 |
| | 71.0 | 7 | 26 | 36 | 6.6 |
| | 67.0 | 7 | 23 | 34 | 5.6 |
| Grimm | 74 | 10 | 29 | 39 | 6.6 |
| | 59 | 25 | 27 | 46 | 5.5 |
| | 17 | 10 | 20 | 118 | 5.6 |
| | | PLANTS IN POROUS CLAY POTS | | | |
| Turkestan | 94 | 8 | 7 | 7 | 3.3 |
| | 84 | 8 | 11 | 13 | 4.4 |
| | 70 | 8 | 31 | 44 | 6.9 |
| | 54 | 8 | 18 | 33 | 5.1 |
| | 48 | 8 | 30 | 62 | 6.0 |
| | 46 | 8 | 23 | 50 | 5.3 |
| | 45 | 8 | 47 | 104 | 9.4 |
| | 25 | 8 | 31 | 124 | 7.2 |
| Grimm | 85 | 8 | 13 | 15 | 4.2 |
| | 73 | 8 | 39 | 54 | 8.9 |
| | 58 | 8 | 11 | 19 | 2.2 |
| | | PLANTS IN POROUS CLAY POTS (CONSTANT MOISTURE) | | | |
| Grimm | 94 | 7 | 7 | 7 | 3.4 |
| | 74 | 8 | 25 | 34 | 6.0 |

It is evident from the data in Table 1 that the coefficient of variability does not offer a dependable measure of the variability of the surviving population, because in general the higher the percentage survival the lower the coefficient of variability. Weinberg's coefficient of variability as used by Winter (7) is not subject to this criticism, since it is not dependent upon the magnitude of the percentage survival of the seedlings after artificial freezing. In comparing the influence of the type of container on the variability of the percentage survival, Weinberg's coefficients were averaged in only those instances in which survival was between 25 and 75 per cent. The data shown in Table 2 indicate that the variations of the variability in percentage survival in the different containers were not large. With Turkestan seedlings there was least variability in the metal flats and cans, while with Grimm there was least in the pots. The metal containers always had a weighed amount of soil which was brought to a constant moisture content before freezing. An attempt was also made to have the moisture content of the soil in the cypress flats and pots as nearly uniform as possible before freezing. With the possible exception of the metal

flats, it appears to make little difference so far as variability in the percentage survival is concerned, which type of container is employed. A serious objection, however, to the use of metal flats is indicated below:

TABLE 2.—*The influence of the various types of containers on the variability in survival of 1-month-old alfalfa seedlings after exposure to low controlled temperatures* [a]

| Kind of alfalfa | Type of container | Average Weinberg's coefficient of variability |
|---|---|---|
| Turkestan | Cypress flats | 5.6 |
| | Metal flats (constant moisture) | 3.9 |
| | Metal cans (constant moisture) | 5.2 |
| | Pots | 6.7 |
| Grimm | Metal cans (constant moisture) | 6.1 |
| | Pots | 5.6 |
| | Pots (constant moisture) | 6.0 |
| Nebraska common | Cypress flats | 5.2 |

[a] Only the data from the experiments reported in Table 1, which showed a survival of 25 to 75 per cent were averaged.

### INFLUENCE OF THE POSITION OF THE ROW IN THE FLATS ON THE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

In order to determine whether the position of the row in the flats had any influence on the survival of seedlings after freezing, the following experiment was undertaken. Seed of Turkestan alfalfa was sown in 6 rows lengthwise in 4 cypress and 4 metal flats. The seedlings were allowed to grow under optimum conditions for one month in the greenhouse before they were transferred to the hardening chamber. Here they remained for two weeks and were then exposed to freezing temperatures. Two weeks later a count of the surviving plants was made. In the cypress flats the percentages of survival were as follows: In the 2 inner rows 87.6, in the 2 intermediate rows 79.9, and in the 2 outer rows 63.9 per cent; in the metal flats the corresponding percentages were 55.8, 35.4, and 19.4. The difference in the percentage survival between the two inner and the two outer rows in the metal flats was much greater (36.4 per cent) than in similar rows in the cypress flats (23.7 per cent).

In order to determine whether there was more variability in survival between plants in the rows of one flat than between rows in different flats, calculations were made from the data obtained in the foregoing study. It was found that the standard deviation calculated by the deviation-from-the-mean method on the basis of six rows within each of 4 cypress flats was 13.9, whereas between rows in the same relative position in different flats it was 20.2. Similar results for the metal flats gave standard deviations of 21.1 and 16.1, respectively. These results show that variability in survival within the same cypress flat is less than that between rows in the same relative position of different flats frozen at the same time. The reverse is true of the variability of survival in the metal flats. The variability within a cypress flat is less than in any of the other combinations. Since it is desirable in comparative cold-resistance tests that comparisons between unknown alfalfas and the control be as accurate as possible, the cypress flats were extensively employed and alfalfas of

unknown hardiness were sown in alternate rows in the same flat with an alfalfa of known hardiness. The percentage survival of the three rows of unknown and the three rows of known alfalfas were averaged, thus eliminating any systematic error with reference to the original position of the seedlings in the flats.

### INFLUENCE OF AGE ON THE RESISTANCE OF SEEDLINGS TO COLD

To determine at what age young alfalfa seedlings would exhibit comparable differences in resistance to cold, the following experiment was undertaken with Turkestan, Grimm, and Arizona common. Triplicate seedings of each alfalfa were made in rows in cypress flats each succeeding week for a period of two months under optimum conditions for growth. At the end of this period all flats were transferred to the hardening chamber maintained at a temperature of 2° to 4° C. and kept there for 15 days. Since a large number of flats were involved, all could not be exposed to low temperatures at the same time, so that it was necessary to freeze on three successive days. The average exposure was six hours at −16°. After freezing, the plants were removed to the greenhouse, and two weeks later survival counts were made. The percentage of survival of the different alfalfas at various ages is shown in Table 3. Apparently, under the conditions of this experiment, seedlings up to the age of 18 days are very susceptible to cold, all alfalfas being almost completely killed out. Comparative differences in cold resistance are well marked whether the seedlings are 25 or 60 days old. The percentage of survival at 32 days is fairly high, although not so high as that of older seedlings.

TABLE 3.—*Influence of age on the cold resistance of alfalfa seedlings*[a]

| Kind of alfalfa | Age of seedlings previous to hardening | Average survival[b] | Average survival of all alfalfas at each age |
|---|---|---|---|
| | *Days* | *Per cent* | *Per cent* |
| Turkestan | 60 | 79 | 51 |
| Grimm | | 54 | |
| Arizona common | | 21 | |
| Turkestan | 53 | 82 | 61 |
| Grimm | | 67 | |
| Arizona common | | 34 | |
| Turkestan | 47 | 51 | 23 |
| Grimm | | 16 | |
| Arizona common | | 1 | |
| Turkestan | 39 | 33 | 20 |
| Grimm | | 26 | |
| Arizona common | | 1 | |
| Turkestan | 32 | 43 | 25 |
| Grimm | | 23 | |
| Arizona common | | 8 | |
| Turkestan | 25 | 23 | 14 |
| Grimm | | 14 | |
| Arizona common | | 6 | |
| Turkestan | 18 | 2 | 1 |
| Grimm | | 0 | |
| Arizona common | | 0 | |
| Turkestan | 11 | 0 | 0 |
| Grimm | | 0 | |
| Arizona common | | 0 | |
| Turkestan | 4 | 0 | 0 |
| Grimm | | 0 | |
| Arizona common | | 0 | |

[a] All plants hardened uniformly for 15 days at 2° to 4° C., and exposed for 6 hours to a temperature of −16° C.

[b] Average of three replications, each replication consisting of 15 to 30 plants of each alfalfa at each stage of development.

Since the freezing in this experiment was too severe to bring out differences in the younger plants, a second experiment was undertaken to ascertain the behavior of the plants at these stages of growth when exposed to less severe freezing. A series of plantings of two alfalfas was made at intervals in pots. At the end of 26 days after the first seeding the entire lot was placed in the hardening chamber and kept there for 15 days. At the end of that time the plants in the pots were exposed for three and one-half hours to a temperature of −14° C. Two weeks later survival counts were made. (Table 4.) Apparently the seedlings were somewhat more resistant to cold in the cotyledon stage than when the third leaf had developed, although there were no appreciable varietal differences in cold resistance at these stages. As the trifoliolate leaves appeared, the seedlings were not only more cold resistant but the varietal response to cold became more marked. It might be mentioned that the stage of development as shown in Table 4 also applies in general to plants of the same approximate age in Table 3. In comparative cold-resistance tests, therefore, the seedlings were grown for approximately one month under optimum conditions, at which time at least three true leaves had developed.

TABLE 4.—*Influence of age and stage of development on the cold resistance of alfalfa seedlings* [a]

| Kind of alfalfa | Age of seedlings previous to hardening | Stage of plant development | Average survival [b] |
|---|---|---|---|
| | *Days* | | *Per cent* |
| Turkestan | 26 | 2 to 3 trifoliolate leaves | 69.0 |
| Arizona common | 26 | ....do.... | 42.0 |
| Turkestan | 19 | 1 trifoliolate leaf | 43.0 |
| Arizona common | 19 | ....do.... | 18.0 |
| Turkestan | 10 | Cotyledons and third leaf | 5.1 |
| Arizona common | 10 | ....do.... | 4.6 |
| Turkestan | 5 | Cotyledons emerging | 13.5 |
| Arizona common | 5 | ....do.... | 13.3 |

[a] All plants in pots hardened uniformly for 15 days at 2° to 4° C., and exposed for 3½ hours to a temperature of −14° C.

[b] Average of 9 replications, each replication consisting of 15 to 30 plants of each alfalfa at each stage of development.

## METHODS OF HARDENING SEEDLINGS

The importance of the hardening process in crop plants has been established through the studies of many investigators. In the present work the writers were interested particularly in determining the optimum condition for hardening and the exposure which would produce maximum hardening of alfalfa seedlings. For these studies the hardening chamber (*3*) was maintained at a uniform temperature of about 2° to 4° C., no attempt being made to vary it.

### INFLUENCE OF THE LENGTH OF EXPOSURE IN THE HARDENING CHAMBER ON THE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

Since the time required for maximum hardening in seedling alfalfas under controlled conditions has apparently never been reported, this constituted one of the essential steps in the development of a comparative test for cold resistance. A number of experiments were undertaken to determine this point.

In the first experiment 50 pots of each of 5 alfalfas—Turkestan, Grimm, Nebraska common, Utah common, and Arizona common—were planted in October and allowed to grow in the greenhouse for 35 days. Four pots of each of the 5 alfalfas, containing from 10 to 20 plants, were then transferred to the hardening chamber, first at 3-day intervals, then at 2-day intervals, and finally at 1-day intervals. All plants of the five alfalfas were then exposed to a temperature of −13.6° C. for four to seven hours. The percentage of plants surviving at the end of two weeks is shown in Table 5, experiment 1. The percentage of survival was highest for the plants that had previously had a 14-day hardening period, and lowest for the plants that had been in the hardening room only 4 to 6 days. In general, the results show that, particularly with the hardier sorts, the longer the plants are held in the hardening room the higher the percentage of survival after artificial freezing and apparently the wider the differences between the alfalfas representing hardy, midhardy, and nonhardy types.

TABLE 5.—*Influence of the length of exposure in the hardening chamber on the survival of alfalfa seedlings to artificial freezing*

| Days in hardening chamber (number) | Percentage survival of— | | | | |
|---|---|---|---|---|---|
| | Turkestan | Grimm | Nebraska common | Utah common | Arizona common |
| **PLANTS IN POTS (EXPERIMENT 1)[a]** | | | | | |
| 14 | 93 | 94 | 75 | 53 | 40 |
| 11 | 69 | 69 | 69 | 72 | 73 |
| 9 | 78 | 67 | 57 | 48 | 29 |
| 7 | 53 | 40 | 22 | 25 | 5 |
| 6 | 45 | 48 | 31 | 26 | 23 |
| 5 | 27 | 15 | 14 | 25 | 8 |
| 4 | 35 | 28 | 7 | 0 | 0 |
| **PLANTS IN POTS (EXPERIMENT 2)[b]** | | | | | |
| 23 | 66 | ..... | ..... | ..... | ..... |
| 19 | 62 | ..... | ..... | ..... | ..... |
| 16 | 65 | ..... | ..... | ..... | ..... |
| 12 | 34 | ..... | ..... | ..... | ..... |
| 8 | 0 | ..... | ..... | ..... | ..... |
| 0 | 0 | ..... | ..... | ..... | ..... |
| **PLANTS IN POTS (EXPERIMENT 3)[b]** | | | | | |
| 35 | ..... | 4 | ..... | ..... | ..... |
| 14 | ..... | 98 | ..... | ..... | ..... |
| 11 | ..... | 80 | ..... | ..... | ..... |
| 6 | ..... | 43 | ..... | ..... | ..... |
| 2 | ..... | 11 | ..... | ..... | ..... |
| **PLANTS IN CYPRESS FLATS (EXPERIMENT 4)[c]** | | | | | |
| 39 | 15 | 18 | 5 | 15 | 1 |
| 18 | 82 | 60 | 58 | 43 | 16 |
| 8 | 61 | 55 | 43 | 20 | 12 |

[a] Each figure represents 4 pots containing 10 to 20 plants of each alfalfa; plants exposed for from 4 to 7 hours at −13.6° C.

[b] Each figure represents 8 pots containing 10 to 20 plants; the Turkestan plants were exposed for 6¾ hours and the Grimm plants for 5½ hours to a temperature of −14.6° C.

[c] Each figure represents three flats containing 5 alfalfas, of about 30 plants each; plants exposed for 8 hours at −17° C.

Only one alfalfa, Turkestan, was employed in the second experiment of this series. Seed was sown in 85 pots at the same time, and eight pots were removed at various intervals to the hardening room. After hardening, the plants were exposed on the same day for 6¾ hours to a temperature of −14.6° C. This temperature was somewhat lower than that employed in experiment 1, and consequently the percentages of survival were lower. The results are shown in experiment 2, Table 5. All the plants that were transferred directly from the greenhouse to the freezer room without hardening succumbed. Even the plants that were held in the hardening chamber for eight days failed to recover. However, four additional days in the hardening chamber resulted in a survival of about 34 per cent. Sixty-five per cent of the plants that had had a hardening period of 16 days recovered. Extending the period up to 23 days apparently did not materially increase the percentage of survival.

A third experiment with Grimm seedlings was similar to the others, except that the length of exposure in the hardening chamber was extended to 35 days. Seed for the plants hardened 35 days were sown a month before the others. The plants were exposed on the same day to a temperature of −14.6° C., but for only 5½ hours. In general, the results are similar to those reported in the preceding experiment, namely, the percentage survival after artificial freezing is directly correlated with the length of the hardening period, within certain limits. It will be noted that only 4 per cent survival resulted when the hardening period was extended to 35 days, and it is apparent that a prolonged period of hardening so weakens the young seedlings that they are easily killed by artificial freezing. In these three experiments the plants, with one exception, were all of the same age at the time of freezing, but they varied in the stage of development, since they were transferred to the hardening chamber at different intervals.

Up to this point the study of the influence of hardening on cold survival under controlled conditions has involved seedlings grown in pots. Although the seedlings were the same age, they had not reached the same stage of development when exposed to artificial freezing, because the different groups of seedlings remained in the hardening chamber for different lengths of time. A fourth experiment was therefore made in which cypress flats were used and the dates of planting were so arranged that the plants would have reached the same stage of development at the time they were transferred to the hardening chamber. Briefly, three flats were sown to each of five alfalfas on three different dates. While the plants were all of the same age when they were placed in the hardening chamber, 1 lot was hardened for 39 days, 1 for 18 days, and 1 for 8 days. The plants in all the flats were then exposed for 8 hours to a temperature of −17° C. The length of exposure was greater and the temperature was lower in this experiment than in the pot experiments, since larger amounts of soil were contained in the flats than in the pots. However, the results are similar to those already given, as may be noted in experiment 4, Table 5. Here again, as in experiment 3, an extended hardening period apparently so weakened the plants that they could not withstand artificial freezing. It is interesting to note that the plants in experiments 1, 2, and 3, Table 5, that were in the hardening chamber longest, were able to overcome

the handicap of being somewhat less advanced in development, which, as Tables 3 and 4 show, would naturally make them less resistant to cold. This fact emphasizes the large degree of hardening which occurs during the longer periods.

From these experiments it may be concluded that 1-month-old alfalfa seedlings not only attain maximum hardening in about 15 days under controlled temperatures of 2° to 4° C., but that these same conditions bring out the greatest differences between the hardiness of the various alfalfas. A hardening period of a few days or one of prolonged duration is not satisfactory, because comparative differences are not so pronounced. Therefore, in comparative hardiness tests 1-month-old seedling alfalfas were held in the hardening chamber for two weeks prior to artificial freezing.

## METHODS OF FREEZING THE SEEDLINGS

For the artificial freezing of alfalfa seedlings a freezer room (*3*) was used in which uniform temperatures at any point between 0° and −30° C. could be maintained for indefinite periods. Before a comparative cold-resistance method could be developed, it was necessary to determine the length of exposure to temperatures which would insure a uniform percentage of survival of the same alfalfa in repeated tests and bring out the maximum differences in survival between alfalfas of different degrees of hardiness.

### INFLUENCE OF THE HARDENING AND FREEZING TEMPERATURES AND THE SOIL TEMPERATURE DURING FREEZING ON PERCENTAGE SURVIVAL OF SEEDLINGS

As the alfalfa seedlings were exposed to low temperatures either in small or large containers, a study of the influence of the various factors involved in determining the correct length of exposure and temperature for a uniform survival of the same alfalfa was necessary. Some of the factors which may be involved are: (1) The soil temperature prior to freezing; (2) the air temperature and length of exposure in the freezer; (3) the rate of freezing of the soil; and (4) the relative importance of the air and soil temperatures on the survival of the seedlings. Flats containing 6 inches of soil and 1-month-old alfalfa seedlings were held at different temperatures prior to freezing. The soil temperature was measured by means of thermocouples at three depths after an exposure for different periods of time and also at two freezer-room temperatures. The results of the various temperature measurements, together with the percentage survival obtained under these conditions, are listed in Table 6. The results show that the soil temperatures previous to freezing have a direct bearing on the degree of lowering of the soil temperatures in the freezer and the subsequent survival of the seedlings. For example, the flats with the high soil temperatures before freezing had a much greater drop in soil temperature than those with a low soil temperature before freezing. This is reflected in the lower percentages of survival. A contributing influence, however, is the degree of hardening which occurred in those plants exposed to the lower temperatures before freezing. This hardening enabled a greater number of plants to survive, even though the final soil temperature was lower than that in the other flats.

TABLE 6.—*Relation of the hardening and freezing temperatures, and the soil temperature during freezing, to the percentage survival of alfalfa seedlings* [a]

| Soil temperature before freezing | Period of exposure | Temperature maintained | Resulting soil temperatures at depths of— | | | Survival of alfalfa plants |
|---|---|---|---|---|---|---|
| | | | 1.3 cm. | 3.8 cm. | 7.6 cm. | |
| ° *C.* | *Hours* | ° *C.* | ° *C.* | ° *C.* | ° *C.* | *Per cent* |
| 19.5 | 2 | −18.3 | 5.3 | 10.0 | 12.0 | 47 |
| 4.0 | 2 | −18.3 | −1.1 | .6 | .9 | 75 |
| 18.0 | 4 | −10.0 | 1.3 | 6.0 | 8.7 | 50 |
| 6.0 | 4 | −10.0 | .4 | 1.1 | 1.7 | 100 |

[a] Based on a study of 4 flats containing 15 to 30 1-month-old seedlings of each of 4 alfalfas.

The air temperatures in the freezer room exert a decided influence on the rate of freezing and the ultimate survival of the seedlings. An air temperature of −18.3° C. for two hours resulted in a 47 per cent survival, whereas a 4-hour exposure at −10° was required to produce approximately the same percentage survival in those flats with a high soil temperature previous to freezing. It will later be shown that the final soil temperature during freezing likewise has an important bearing on the subsequent survival of the seedlings. In other words, all these factors are important and each must be considered in determining the proper procedure in comparative freezing tests.

### INFLUENCE OF THE PRECENTAGE OE SOIL MOISTURE ON THE RATE OF FREEZING IN THE SOIL AND PERCENTAGE SURVIVAL OF SEEDLINGS

In the preceding section it was shown that soil temperatures at the time the flats were placed in the freezer influenced the rate of freezing the soil. In these experiments moisture content of the soil was uniformly high. In order to determine the influence of the percentage of soil moisture on the rate of freezing, four alfalfas (Grimm, Nebraska common, New Mexico common, and California common) were sown in each of four flats and allowed to grow for one month under optimum conditions. They were then transferred to the hardening chamber for a period of two weeks. Just before freezing, the soil was allowed to dry out, so that it contained approximately 12 per cent moisture in all the flats. The soil in three flats was then brought to 17, 27, and 33 per cent moisture, and the soil in the fourth allowed to remain at 12 per cent. The flats were then exposed for various periods to an average temperature of −18.9° C.

By means of thermocouples placed in the soil at a depth of 1.3 centimeters the rate of depression of the soil temperature was obtained. After freezing, the flats were transferred to the greenhouse, and two weeks later survival counts were made. The results of this experiment are presented graphically in Figure 1. In the soil with a moisture content of 12 and 17 per cent the temperatures dropped to 0° C. within a few minutes, and within four hours had reached temperatures of −5° and −4°, respectively. On the other hand, in the soil with a moisture content of 27 per cent, the temperature at the end of 4 hours was still above 0° and it did not reach −4° until after a 10-hour exposure, a difference of 6 hours. Finally, in the flat having a soil moisture of 33 per cent, the temperature did not reach 0°

until after a 5-hour exposure, and from this point the temperature gradually dropped to −2.2° at the end of over 15 hours.

Thus the drier the soil, the faster was the temperature depressed. The higher the moisture content of the soil, the more gradual was the temperature depression. The lowest temperatures were also recorded in the drier soil. In spite of the shorter exposure, the percentages of survival were lower in the dry soil and progressively higher as the moisture content of the soil increased.

These results show that the influence of the moisture content of the soil is pronounced, not only on the rate and final temperature depression in the soil, but also on the percentage survival of the plants. Thus, in comparative hardiness tests with seedling alfalfas

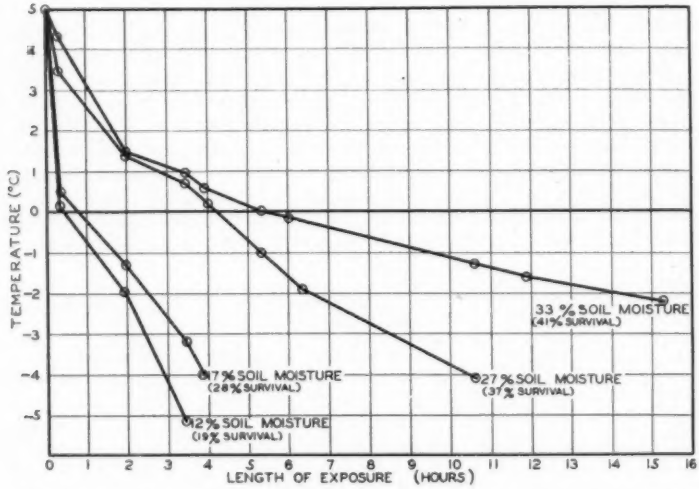


FIGURE 1.—Rate of temperature depression in the soil, as influenced by the soil-moisture content, and subsequent percentages of survival of alfalfa seedlings exposed in flats to a temperature of −18.9° C. for different periods of time. The 1-month-old seedlings were hardened for two weeks at the same soil moisture, which was adjusted just prior to freezing

it is imperative that not only the temperature of the soil but also the moisture content be made as uniform as possible before the seedlings are frozen. A comparatively high soil moisture is to be preferred because a more uniform and gradual temperature depression occurs and the exposure can be extended over longer periods. In all comparative hardiness tests the moisture of the soil in which the seedlings are growing is made relatively high and uniform in all flats exposed at one time in the freezer room.

## INFLUENCE OF LENGTH OF EXPOSURE TO LOW TEMPERATURES ON PERCENTAGE SURVIVAL OF SEEDLINGS

The technic of freezing seedlings has not been so perfected that it is possible to state that an exposure to a stated low temperature for a definite period will result in a certain percentage of survival. Neither have enough records been accumulated to show whether a short exposure to a low temperature will give more uniform and

consistent results than a long exposure to a higher temperature. Numerous trials have indicated that records of the soil temperature depressions aid greatly in determining the proper freezing exposure. Better results have been obtained when the containers were exposed for relatively short intervals at a low temperature than when they were left until the soil had reached the air temperature of the freezer room. This necessitates longer exposures at higher temperatures. The former procedure is more nearly comparable to conditions in the field, where mininum soil temperatures rarely approach minimum air temperatures. Therefore, in most of the comparative hardiness trials temperatures between $-10°$ and $-20°$ C. were employed, with relatively short exposures. No definite freezing exposure can be given, as this will vary with the degree of hardening of the seedlings as well as with soil type and other factors.

The length of exposure to a given low temperature, however, is to some extent reflected in the percentage of survival, as shown in Table 7. The percentages of survival were obtained by averaging the number of 1-month-old plants in five pots of each of eight alfalfas, hardened off for a 2-week period, and exposed for various intervals to a temperature of $-14.5°$ C. An hour and twenty minutes of exposure to this temperature resulted in a survival of 72 per cent, while an exposure of 3½ hours reduced the survival to 20 per cent. For each increase in time of exposure between these two points there was a definite decrease in the number of seedlings that survived.

TABLE 7.—*Influence of the length of exposure to a temperature of $-14.5°$ C.[a] on the percentage survival of alfalfa seedlings*

| Length of exposure | | Average survival of alfalfa plants |
|---|---|---|
| *Hours* | *Minutes* | *Per cent* |
| 1 | 20 | 72 |
| 1 | 50 | 61 |
| 3 | 00 | 33 |
| 3 | 30 | 20 |

[a] Based on a study of 5 pots containing approximately fifteen 1-month-old plants, of each of 8 alfalfas, i. e., 600 plants. The plants had been hardened for 15 days.

## METHOD OF HANDLING THE SEEDLINGS AFTER FREEZING

In practically all the experiments reported, the plants were removed directly to the greenhouse from the freezer room. From 24 to 36 hours elapsed before the soil in the flats came to the greenhouse temperature. Investigators are still in disagreement as to whether the rate of thawing is a factor in the differential responses of alfalfas after artificial freezing. Janssen (*2*) states that slow thawing increases the percentage of survival in wheat; Weimer (*6*) reports that slow thawing does not increase the percentage survival in alfalfa. The writers (*5*) have presented data which show that slow thawing of 2-year-old alfalfa plants increased the percentage of survival.

In an experiment one set of seedlings in pots was returned directly to the hardening chamber from the freezer room and held for a period of eight days before removal to the warm greenhouse, while a second set was taken immediately from the freezer room to the greenhouse.

At the end of two weeks the average percentage survival of those held in the hardening chamber for eight days and thawed slowly was $32 \pm 1.9$ per cent as compared to $18 \pm 1.5$ per cent for those which were thawed rapidly. Good comparable results were obtained by following a uniform practice in removing the plants directly from the freezer to the greenhouse. It yet remains to be determined whether there is a differential varietal response to slow thawing which would serve to bring out greater differences.

## APPLICATION OF THE METHOD IN COMPARATIVE COLD RESISTANCE TESTS WITH ALFALFA

In developing methods for testing comparative cold resistance, a number of alfalfas, representing different degrees of hardiness, were employed in order to compare the results with those that would be expected under field conditions with the same sorts of alfalfa. As a rule the kinds used had been tested in general agronomic practice for a period of years, so their hardiness was known.

In the first tests three alfalfas of different degrees of hardiness were employed. Each alfalfa was grown in 24 replications in rows in flats, so that there were approximately 480 plants of each alfalfa. The seedlings were grown for 1 month in the greenhouse, transferred to the hardening chamber for 13 days, and then exposed to a temperature of $-19.6°$ C. for 8½ hours. The percentages of survival are listed in Table 8. They are significantly different and in the same order as shown in field tests.

TABLE 8.—*Comparative cold resistance of alfalfas* [a]

| Kind of alfalfa | Survival of plants | |
|---|---|---|
| | *Per cent* | *P. E.* [b] |
| Turkestan (F. C. I. No. 15754) | 40 | ±3.4 |
| Nebraska common | 27 | ±2.8 |
| Arizona (F. C. I. No. 15837) | 3 | ±.7 |

[a] Based on a study of 24 replications of 15 to 30 plants per row. Plants were grown for 1 month in flats, hardened for 13 days, and exposed for 8½ hours to a temperature of −19.6° C.
[b] P. E. of the mean, calculated by deviation-from-the-mean method.

In a second experiment five alfalfas were employed, and seed was sown in pots and in rows in flats. Each alfalfa was replicated six times with 15 to 30 plants in each row in the flats, and twenty times with each 10 to 20 plants in each pot. The plants in the two types of containers were grown in the greenhouse for 1 month, transferred to the hardening chamber for 15 days, and finally the flats were exposed for 6 hours to a temperature of $-16.0°$ C. and the pots for 5½ hours to a temperature of $-13.8°$. Two weeks after freezing, survival counts were made. (Table 9.) In general they give a relative percentage of survival comparable to that obtained in field tests. While the percentages of survival in the two types of containers are not always identical, because of different lengths of exposure to low temperatures, the results are in agreement in differentiating the degree of hardiness between alfalfas. Some may question the placing of Turkestan above Grimm in the hardiness scale. However, these results have been consistent in the writer's tests. The writers (4) have shown that certain lots of Turkestan are more hardy under the conditions of these tests than Grimm, whereas some are less hardy.

TABLE 9.—*Comparative cold resistance of alfalfas* [a]

| Kind of alfalfa | Survival of plants— | | | |
|---|---|---|---|---|
| | In flats | | In pots | |
| | *Per cent* | *P. E.* [b] | *Per cent* | *P. E.* [b] |
| Turkestan (F. C. I. No. 15754) | 72 | ±7.3 | 70 | ±4.5 |
| Grimm (F. C. I. No. 15713) | 58 | ±5.9 | 68 | ±5.3 |
| Nebraksa common | 51 | ±5.2 | 53 | ±5.6 |
| Utah (F. C. I. No. 15815) | 32 | ±3.3 | 50 | ±5.9 |
| Arizona (F. C. I. No. 15837) | 14 | ±1.4 | 40 | ±5.7 |

[a] Based on average results from 6 flats, each flat containing 15 to 30 plants of each alfalfa, and from 20 pots containing 10 to 20 plants. Plants were grown for 1 month, hardened for 15 days; flats were exposed for 6 hours to a temperature of −16.0° C. and pots for 5½ hours to −13.8° C.
[b] P. E. of the mean, calculated by deviation-from-the-mean method.

In order to test the validity of the method further, a series of eight alfalfas of known hardiness were sown both in pots and in rows in cypress flats. The seedlings were allowed to grow for one month in the greenhouse. Those in the flats were then hardened for 13 days and those in the pots for 11 days. The flats were then removed to the freezer room and exposed for 2⅓ hours to a temperature of −20.4° C. To insure success in obtaining the correct freezing exposure, the pots were divided into four lots of five pots each for each alfalfa. The first lot was exposed for 1⅓ hours, the second for 1⅝, the third for 3, and the fourth for 3½ hours to a temperature of −14.4°.

The percentage of seedlings surviving after these exposures in the freezer room are given in Table 10, together with the probable errors, calculated by the deviation-from-the-mean method. The higher probable errors in the tests with flats can be accounted for by the fact there were only four replications, whereas with the pots there were 20. This experiment, therefore, gives some idea of the number of replications necessary for comparative testing for cold resistance of alfalfas.

TABLE 10.—*Comparative cold resistance of alfalfas*

| Kind of alfalfa | F. C. I. accession No. | Survival of plants— | | | |
|---|---|---|---|---|---|
| | | In flats [a] | | In pots [b] | |
| | | Per cent | P. E. [c] | Per cent | P. E. [c] |
| Ladak | 14135 | 47 | ±8.9 | 78 | ±1.9 |
| Turkestan | 15754 | 46 | ±8.7 | 65 | ±1.6 |
| South Dakota common | 14210 | 22 | ±4.2 | 56 | ±2.6 |
| Kansas common | 15749 | 8 | ±1.5 | 45 | ±3.5 |
| New Mexico common | 14470 | 3 | ± .6 | 42 | ±2.5 |
| Provence | 15744 | 2 | ± .4 | 29 | ±2.0 |
| Arizona | 15837 | 1 | ± .2 | 33 | ±2.3 |
| Peruvian | 15830 | 0 | ..... | 24 | ±1.5 |

[a] Based on 4 replications of 15 to 30 1-month-old plants for each alfalfa, hardened for 13 days, and exposed for 2⅓ hours to a temperature of −20.4° C.
[b] Based on 20 replications of 10 to 20 1-month-old plants for each alfalfa, hardened for 11 days, and exposed for from 1⅓ to 3½ hours to a temperature of −14.4° C.
[c] P. E. of the mean, calculated by deviation-from-the-mean method.

The agreement between the rank of the alfalfas tested in the flats and pots is very close, and corresponds very nearly with the relative hardiness found with these same alfalfas in field experiments. During the past season (1930–31), individual hardiness tests have been made on 100 different sorts of alfalfa and, in general, as judged by

those whose hardiness was known, the results have been uniform and consistent in giving an expression of their relative hardiness.

With a sufficiently large number of replications, the results obtained indicate the possibility of a wide application of this method. In addition to its adaptability for testing new strains, selections, and introductions, it would appear to be valuable for selecting hardier types within a mass population.

## SUMMARY

The steps involved in the development of a method for the determination of comparative hardiness in seedling alfalfas under controlled conditions have been presented. This method as developed up to the present time consists essentially of the following general procedure.

Alfalfas are seeded in small pots or preferably in cypress flats in alternate rows with a control alfalfa of known hardiness, and allowed to grow under optimum conditions in the greenhouse for one month. They are then transferred to the hardening chamber, held at a temperature of 2° to 4° C. for two weeks. Before the seedlings are frozen, the soil is brought to a high and uniform moisture content. The flats with the seedlings are then exposed in the freezer room for a number of hours to a temperature at some point between −10° and −20°. The length of exposure to low temperatures is so gauged that about 50 per cent of the control alfalfa survives. After freezing, the seedlings are removed to the greenhouse and two weeks later survival counts are made. The actual percentages of survival of the alfalfas are calculated in terms of the control alfalfa, and comparisons between alfalfas are made by this standard.

This method gives reliable and consistent results in the determination of relative hardiness in different alfalfas, and offers a rapid means whereby they can be tested for comparative cold resistance. This method may also be used for selecting hardier types within a strain or variety of alfalfa.

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# INHERITANCE IN BARLEY[1]

By D. W. ROBERTSON, *Associate Agronomist*, G. W. DEMING, *Assistant in Agronomy*, and DWIGHT KOONCE, *Assistant in Agronomy*, *Colorado Agricultural Experiment Station*[2]

## INTRODUCTION

Although an abundance of genetic work has been done on animals, only a few plant species have been studied in detail. Barley, a crop of economic importance, has many easily determinable characters and a small number of chromosomes. For this reason it offers good material for inheritance studies. With the common occurrence of chlorophyll-defective seedlings the chance of identifying different character pairs has increased, and a large number of factor pairs has been added to those already known. The studies presented in this paper are a continuation of previous studies made for the purpose of establishing linkage groups in barley.

## REVIEW OF LITERATURE

A fairly extensive review of the literature on linkage relations in barley is now available (*5*, *6*, *3*, *1*, *2*).[3] The character pairs discussed in this paper which have been studied by previous workers are shown in Table 1.

TABLE 1.—*Character pairs discussed in this paper which have been studied by previous workers*

| Character pair | Investigator | Number of factors involved | Symbol used |
|---|---|---|---|
| Rough v. smooth awn | Hayes and Garber (*5*) | Single-factor difference | (*Rr*). |
| | Griffee (*3*) | 2-factor difference. $F_2$ ratio, 12:3:1. | (*RrSs*). |
| | Sigfusson (*12*) | 2-factor difference. $F_2$ ratio, 9:3:3:1. | (*RrSs*). |
| Style branching | Robertson and Deming (*11*). | 3 pairs of cumulative factors. | *Gg*, *G'g'*, *G''g''*. |
| Long v. short haired rachilla | Hor (*7*) | Single-factor difference | (*Ll*). |
| | Robertson (*10*) | | (*Ss*). |
| | Sigfusson (*12*) | | (*Ll*). |
| | Buckley (*1*) | | (*Ll*). |
| Black v. white glume color | Griffee (*3*) | do | (*Bb*). |
| | Hayes and Garber (*6*) | | (*Bb*). |
| | Robertson (*10*) | | (*Bb*). |
| | Sigfusson (*12*) | | (*Bb*). |
| Blue v. white aleurone | Sō and Imai (*13*) | Single-factor difference (xenia). | (*Bl bl*). |
| | Hays and Garber (*5*) | | |
| | Buckley (*1*) | | |
| Hoods v. awns | Hor (*7*) | Single-factor difference | (*Kk*). |
| | Hayes and Garber (*5*) | | (*Kk*). |
| | Robertson (*10*) | | (*Kk*). |
| | Buckley (*1*) | | (*Kk*). |
| Green v. chlorina | Nilsson-Ehle (*9*) | do | (*Ff*). |
| | Hallqvist (*4*) | | (*Ff*). |
| Green v. albino $_3$ | Nilsson-Ehle (*9*) | do | ($As_{42}$) |
| | Hallqvist (*4*) | | ($As_{42}$). |

[1] Received for publication Aug. 8, 1931; issued April, 1932. The part of this paper dealing with the character differences in a Coast × Lion cross was submitted to the Department of Agronomy, Utah State Agricultural College, by Dwight Koonce in partial fulfillment of the requirements for the degree of master of science, March, 1931.

[2] The writers gratefully acknowledge the cooperation of Director C. P. Gillette and Prof. Alvin Kezer, of this station, in providing facilities for this investigation. The writers are also indebted to Dr. H. K. Hayes, professor of plant genetics, University of Minnesota, for helpful criticisms of the manuscript.

[3] Reference is made by number (italic) to Literature Cited, p. 466.

TABLE 1.—*Character pairs discussed in this paper which have been studied by previous workers*—Continued

| Character pair | Investigator | Number of factors involved | Symbol used |
|---|---|---|---|
| Green v. albino | Hallquist (*4*) | Single-factor, difference | ($A_1a_4$). |
| Green v. albino | Robertson (*10*) | do | ($A_2a_2$). |
| Green v. xantha | do | do | ($X_2x_2$). |
| Green v. albino | do | do | ($A_3a_3$). |
| Do | Robertson and Deming (*11*). | do | ($A_{c2}a_{c2}$). |
| Green v. yellow | do | do | ($X_{c2}x_{c2}$). |
| Green v. virescent | do | do | ($Y_{c2}y_{c2}$). |
| Green v. chlorina | do | do | ($F_{c2}f_{c2}$). |

Three types of $F_2$ segregations have been described in differentiating between rough and smooth awns. Hayes and Garber (*5*), in summarizing previous data, report that the $F_1$ plants in crosses between rough and smooth awned varieties have rough awns, while the $F_2$ generation segregated into rough and smooth awned plants in an approximate 3:1 ratio. Griffee (*3*) and Sigfusson (*12*) report a 2-factor difference. Griffee was able to divide the $F_2$ plants into three phenotypes only based on an arbitrary awn index division. The types rough, intermediate smooth, and smooth were obtained in a 12:3:1 ratio. The factor *R* produces rough awns, while the factor *S* is hypostatic to *R* and in the absence of *R* produces intermediate-smooth awns. The double recessive *rr ss* produces smooth awns. Sigfusson classifies the $F_2$ plants into four groups according to the degree of roughness. He states (*12, p. 666*):

> The rough and intermediate rough classes have barbs the entire length of the awn, but the awns of the latter class are not nearly as scabrous. When heads of these phenotypes were examined in sunlight, and when held at the correct distance from the eye to be properly focussed, the difference could be easily discerned.

Sigfusson classified the $F_2$ as rough, intermediate rough, intermediate smooth, and smooth. A close approximation, of a 9:3:3:1 ratio was obtained. The factor *R*, either single or in duplicate and in the absence of *S*, produced the intermediate-rough condition, and likewise the factor *S*, in the absence of *R*, produced the intermediate-smooth condition. Both factors are necessary to produce the fully barbed condition. The double recessive *rr ss* produced the smooth-awn class.

Daane (*2*) reviews the previous work on linkage and describes five linkage groups: (1) The non 6-rowed versus 6-rowed character pair; (2) black versus white lemma and pericarp; (3) hulled versus naked seed; (4) hooded versus awned; and (5) rough versus smooth awn. Several other characters have been found which have not yet been placed in any of the linkage groups described.

Hor (*7*) reports a linkage between black versus white glume color, rough versus smooth awn, and long versus short haired rachilla.

Robertson (*10*), Sigfusson (*12*), and Buckley (*1*) found the factor pairs for black versus white glume color and rough versus smooth awn to be inherited independently.

Sigfusson (*12*) and Hor (*7*) obtained a linkage between long versus short haired rachilla and one of the factors for rough versus smooth awn. Sigfusson gives a crossover percentage of 30.8. Hor found a

crossover percentage of $28.70 \pm 3.43$ for the repulsion phase and $34.54 \pm 2.89$ for the coupling phase.

Buckley (*1*) reports a linkage of the factor pairs *Bl bl* for blue versus white aleurone and *Kk* for hoods versus awns. A minimum $\chi^2$ value of 2.23 was obtained at a crossover value of 41 per cent.

Hallquist (*4*) reports a linkage of the following chlorophyll-defective seedling factors: $\text{Albino}_4$, $\text{albino}_3$, and chlorina. The crossover percentages were found to be 10.2 for $\text{albino}_3$ and chlorina; 3.8 for chlorina and $\text{albino}_4$; and 12.5 for $\text{albino}_3$ and $\text{albino}_4$.

## VARIETIES USED IN THE EXPERIMENTS

This paper presents a study of the inheritance of various factor pairs and their possible linkage relations. The following varieties were used in the various studies: Coast C. I. No. 2791, Lion C. I. No. 923, Minnesota 84–7, Trebi, Coast III, Colsess I, Colsess IV, Colsess V, and Minnesota 72–8.

Coast C. I. No. 2791 has a white glume, short-haired rachilla, blue aleurone, branched styles, and rough awn. The barbing extends the full length of the awn. The grain is hulled.

Lion C. I. No. 923 has black glumes, long-haired rachilla, unbranched styles, and smooth awns. However, there is barbing of the awns at the base, which was disregarded in this study. Also, there is some barbing on the tip of the awns which varies, as is shown by the awn indices.

Minnesota 84–7 is a 2-rowed, white-hulled, awned barley. The rachilla hairs are long and it carries the factor pair *ff* for chlorina plant color. This barley was obtained from the Minnesota station and came originally from C. Hallquist. Nilsson-Ehle (*9*) describes it as a pale-green chlorophyll-deficient type. It is a 2-rowed strain of Gold. The color of the seedlings is "cosse green" (Ridgway, Pl. V).[4] The plants grow to maturity but are somewhat stunted.

Trebi (*10*) is a 6-rowed, bearded, hulled barley with heads very similar to those of Coast. The strain Trebi I carries a factor pair ($A_t a_t$) for green versus white seedlings which has previously been found linked with the factor pair *Bb* for black versus white glume color.

Coast III is similar to Coast, but carries a factor pair ($Y_c y_c$) for green versus virescent seedlings (*11*). The virescent seedling dies in the seedling stage. It comes up with a very marked green tip on the first leaf.

Colsess is a 6-rowed hooded barley with a hulled grain of a bluish-green color. The straw and glume are light yellow. The shank of the hood is about 5 mm long and is barbed at the base. The rachis is rather tough and the head does not shatter easily. The rachilla hairs are short and the outer glume is covered with very short hairs. Several strains of this variety have been used in the studies of chlorophyll deficiencies. Colsess I and Colsess IV (*10*) carry the factor pair $A_c a_c$ for green versus white seedlings and Colsess IV carries the factor pair $X_c x_c$ for green versus xantha seedlings. The factor pairs $A_c a_c$ and $X_c x_c$ have previously been found to be closely linked. The strain Colsess V (*11*) is a chlorina plant, less vigorous than the normal

[4] RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., illus., Washington, D. C. 1912.

green plants, and lighter in color. The plants are "dull green-yellow" (Ridgway, Plate XVII).[5]

Minnesota 72–8 is a 6-rowed, hulled, awned barley, obtained from the Minnesota station. The original plant came from Hallquist and has the factor pair *Yy* for green versus virescent seedlings. The virescent seedlings have a slightly green tip, but fail to survive beyond the seedling stage.

Methods similar to those used in previous work by the senior author(*10*) were employed.

The strains containing the chlorina factor pairs *Ff* and $F_cf_c$ were recessive for the factors for chlorina. These chlorina plants were used as parents instead of plants heterozygous for the chlorophyll deficiencies used in previous studies.

## INHERITANCE OF SIMPLE MENDELIAN CHARACTERS

### CHLORINA SEEDLINGS IN MINNESOTA 84–7

The inheritance of the chlorina seedlings in Minnesota 84–7 was studied in a cross between chlorina plants of Minnesota 84–7 and Trebi plants heterozygous for the factor pair for green versus white seedlings $A_tA_t$. All of the $F_1$ plants were green, indicating that the factor pairs *Ff* and $A_tA_t$ are not allelomorphic. There were 9 $F_1$ plants which segregated in $F_2$ for green and chlorina plants and 14 which segregated for green, chlorina and white. The segregation of the green and chlorina plants indicates a single-factor difference. Table 2 presents the data obtained from these crosses.

TABLE 2.—$F_2$ *segregation of green and chlorina seedlings in Minnesota 84–7 × Trebi*

| Item | Green | Chlorina | Deviation | D/PE |
|---|---|---|---|---|
| Observed count | 1,453 | 512 | | |
| Calculated segregation, 3:1 | 1,473.75 | 491.25 | 20.75 | 1.6 |

### VIRESCENT SEEDLINGS IN MINNESOTA 72–8

The mode of inheritance of green and virescent seedlings was studied in a cross between Colsess IV, heterozygous for the factor pair $X_cx_c$ for green versus xantha seedlings and Minnesota 72–8, heterozygous for the factor pair *Yy* for green versus virescent seedlings. The number of green and virescent seedlings obtained from the progeny of six $F_1$ plants which were heterozygous for the seedling factor pair *Yy* is shown in Table 3.

TABLE 3.—*$F_2$ segregation of green and virescent seedlings from plants heterozygous for the green and virescent factor pair (Yy) in Colsess IV × Minnesota 72–8*

| Item | Green | Virescent | Deviation | D/PE |
|---|---|---|---|---|
| Observed count | 926 | 327 | | |
| Calculated segregation, 3:1 | 939.75 | 313.25 | 13.75 | 1.33 |

The segregation of green and virescent seedlings indicates a single-factor difference.

[5] RIDGWAY, R. Op. cit.

### BLUE VERSUS WHITE ALEURONE (Bl Bl)

The inheritance of blue and white aleurone was studied in a cross between Colsess IV and Minnesota 72–8. A separation of the blue and white aleurone color was attempted in the $F_1$ plants, but some difficulty was encountered, especially with small immature seeds. The $F_2$ plants, however, were separated in the field into homozygous blue aleurone, heterozygous plants having both blue and white seeds on the same head, and homozygous white aleurone. When the plants were grouped into two groups, those containing homozygous and heterozygous blue aleurone and those with colorless aleurone, the segregations shown in Table 4 were obtained.

TABLE 4.—*$F_2$ segregation of seeds with colored aleurone and colorless aleurone, as determined from $F_2$ plants, in Colsess IV × Minnesota 72–8*

| Item | Colored | Colorless | Deviation | D/PE |
|---|---|---|---|---|
| Observed count | 4,553 | 1,395 | | |
| Calculated segregation, 3 : 1 | 4,461 | 1,487 | 92 | 4.09 |

The number of plants with colorless aleurone is small. When plants are grouped as homozygous blue and heterozygous blue and colorless, a much better fit to the calculated 3 : 1 ratio is obtained. Table 5 gives the grouping of the different $F_2$ seeds.

TABLE 5.—*$F_2$ segregation of seeds with pure blue aleurone (Bl Bl) and nonpure blue aleurone (Bl bl and bl bl) as determined from the $F_2$ plants in Colsess IV × Minnesota 72–8*

| Item | Heterozygous blue and colorless | Homozygous | Deviation | D/PE |
|---|---|---|---|---|
| Observed count | 4,420 | 1,528 | | |
| Calculated segregation, 3 : 1 | 4,461 | 1,487 | 41 | 1.82 |

The results shown in Tables 4 and 5 may be explained as due to a single-factor pair.

The better fit in the classification of nonblue and blue-seeded plants may be explained by the fact that white seeds on $F_2$ plants may have been fertilized with pollen carrying the factor for blue, and all plants showing any blue aleurone in the heads were classified as heterozygous. The progeny of some $F_2$ white seeds ($F_1$ plants) evidently were classified as heterozygous instead of colorless, which reduced the number of colorless plants.

### ROUGH VERSUS SMOOTH AWN

The inheritance of rough versus smooth awns was studied in a Coast × Lion cross. The $F_2$ plants were classified into three groups, rough, intermediate smooth, and smooth. The method used was the same as that employed by Hayes et al. (*6*) and Griffee (*3*). An awn of average length was taken from the center of the main spike of each plant and examined under the microscope. Hayes et al. (*6*) state:

> The distance on the tip of the awn upon which teeth were regularly borne was measured. The total length of the awn was divided by the length of the tip upon which teeth were found. The result obtained was called the awn index; the larger the index, the smoother the awn and vice versa.

Coast and the rough $F_2$ plants have an awn index of 1. The awn indices of 100 plants of the Lion parent were determined. The awn indices of this group ranged from 2.7 to 7.6, with an average of 4.3. A division of the partially smooth plants was made at the awn index of 2.7, which was the lower limit of the Lion plants. The plants with an awn index of 2.7 or more were classified as smooth, and the plants with an awn index of 1.1 to and including 2.6 were classified as intermediate smooth.

As shown in Table 6, the observed segregation approaches a 12:3:1 ratio. By applying the test for goodness of fit, a $\chi^2$ value of 2.28 was obtained with a $P$ value of 0.3273. This segregation is similar to that found by Griffee (*3*), and can best be explained on the basis of 2-factor difference for roughness of awn. $R$ and $R'$ are used to designate the factors for roughness of awn, and $r$ and $r'$ denote the absence of the rough condition. When the factor $R$ is present the awn is rough. $R'$ is hypostatic to $R$, and in the absence of $R$ gives intermediate smooth awned plants. The double recessive, $rr\ r'r'$, gives the smooth group similar to the Lion parent.

TABLE 6.—*Segregation in the $F_2$ generation for rough, intermediate smooth, and smooth awns in Coast×Lion*

| Item | Rough [a] | Intermediate smooth [a] | Smooth [a] |
|---|---|---|---|
| Observed count | 852 | 191 | 61 |
| Calculated segregation, 12:3:1 | 830.25 | 207.56 | 69.19 |

$\chi^2=2.28$. $P=0.3273$.

[a] Awn indices: Rough, 1; intermediate smooth, 1.1 to 2.6; smooth, 2.7 or over.

## BRANCHING OF STYLE

In a previous paper, Robertson and Deming (*11*), have reported a 3-factor difference between the smooth style of Lion and the feathered style of Coast. The factor pairs are designated $Gg\ G'g'\ G''g''$. The behavior of about 50 $F_3$ plants from each $F_2$ family was studied. With independent inheritance of three cumulative factors, the following ratio of differently segregating plants would be expected in the $F_3$ generation. Thirty-seven plants would have one or more of the factors in the homozygous dominant condition and would give plants with some degree of branching on the style, 12 plants would segregate 15 branched to 1 unbranched, 8 would segregate 63 branched to 1 unbranched, 6 would segregate 3 branched to 1 unbranched, and 1 would breed true for the unbranched condition.

The following data were obtained from the $F_3$ families studied. Forty-five $F_3$ families segregated 15:1, 35 segregated 3:1, 17 segregated 63:1, and all of the $F_2$ plants with unbranched styles bred true. A poor fit to the calculated number of segregating families was obtained. Some error evidently crept in from the small number of plants in the $F_3$ families, and possibly this would account for the small number of families segregating 63:1. However, when we consider the close fit of the $F_2$ segregation to the calculated 63:1 ratio and the fact that all the calculated $F_3$ ratios were obtained, it may be concluded that these results are best explained on a 3-factor hypothesis. These factors are cumulative in their effect, and it is necessary for all

the factors to be recessive in order to produce the unbranched styles of the Lion type.

## INTERRELATIONSHIP OF CHLOROPHYLL DEFICIENCIES

### GREEN VERSUS CHLORINA (Ff) AND GREEN VERSUS WHITE SEEDLINGS ($A_tA_t$)

The interrelationship of the factor pairs *Ff* and $A_tA_t$ was studied in a cross between Minnesota 84–7 homozygous for chlorina (*ff*) and Trebi, heterozygous for green and white seedlings ($A_tA_t$). All the $F_1$ plants were green, indicating that the factor pairs *Ff* and $A_tA_t$ are not allelomorphic. There were 9 $F_1$ plants that segregated in $F_2$ for green and chlorina and 14 that segregated for green, chlorina, and white. The interrelationship of the factor pairs *Ff* and $A_tA_t$ was studied in the progeny of the 14 plants that segregated for green, chlorina, and white seedlings. (Table 7.)

TABLE 7.—*$F_2$ segregation of green, chlorina, and white seedlings in Minnesota 84–7 × Trebi*

| Item | Green | Chlorina | White |
|---|---|---|---|
| Observed count | 3,333 | 1,164 | 1,554 |
| Calculated segregation, 9:3:4 | 3,403.7 | 1,134.6 | 1,512.7 |

$\chi^2=3.3579$. $P=0.1917$.

The test for goodness of fit to a 9:3:4 ratio gave a *P* value of 0.1917, indicating independent inheritance of the factor pairs $A_tA_t$ and *Ff*.

### GREEN VERSUS CHLORINA (Ff) AND GREEN VERSUS WHITE SEEDLINGS ($A_cA_c$)

The inheritance of green versus chlorina (*Ff*) and green versus white seedlings ($A_cA_c$) was studied in a cross between Minnesota 84–7 and Colsess. The following crosses were grown in $F_1$ and $F_2$: II–26–440, II–26–421, II–27–148, and II–27–151. All the $F_1$ plants were green, indicating that the factor pair for green versus chlorina (*Ff*) and the factor pair for green versus white in Colsess ($A_cA_c$) are not allelomorphs. From a cross of the above types, where a pure chlorina plant *ff* is crossed with a plant heterozygous for green and white seedlings $A_cA_c$, only two kinds of $F_1$ plants would be expected, namely, those giving progeny segregating in $F_2$ into green and chlorina seedlings and those segregating into green, chlorina, and white seedlings. The progeny of 10 plants of the former type and 7 of the latter were studied.

The interrelationship of the factor pairs *Ff* for green versus chlorina seedlings and $A_cA_c$ for green versus white seedlings was studied in the families that segregated for all three types of seedlings. The following segregations were obtained: Crosses II–27–148 and II–27–151 produced 1,397 green, 541 chlorina, and 599 white seedlings. The number of chlorina plants is greater than the calculated 9:3:4 ratio. A similar but wider variation was found in cross II–26–421. The $F_2$ plants segregated into 776 green, 302 chlorina, and 308 white. In order to test the possibility of linkage, the $F_3$ segregation from the $F_2$ green plants was determined. (Table 8.)

TABLE 8.—*Genotypes of $F_2$ green plants as determined from $F_3$ seedling counts in various crosses*

| Cross No. | Number of plants having indicated genotype | | | | | |
|---|---|---|---|---|---|---|
| | $FFA_cA_c$ | $FfA_cA_c$ | $FFA_ca_c$ | $FfA_ca_c$ | $\chi^2$ | $P$ |
| II–26–440 | 76 | 170 | 152 | 331 | 1.4722 | 0.6932 |
| II–26–421 | 35 | 80 | 79 | 174 | 1.6476 | .6530 |
| II–27–148 and 151 | 133 | 265 | 218 | 522 | 6.2535 | .1015 |

The data in Table 8 indicate independent inheritance of the factor pairs *Ff* for green versus chlorina seedlings and $A_ca_c$ for green versus white seedlings.

GREEN VERSUS VIRESCENT SEEDLINGS $Y_cy_c$ AND GREEN VERSUS CHLORINA SEEDLINGS $F_cf_c$

The relationship of the factor pairs concerned was studied in a cross between Coast, heterozygous for green and virescent seedlings $Y_cy_c$ and Colsess, homozygous for chlorina seedlings $f_cf_c$.

Two types of segregating progeny were obtained. Eleven $F_1$ plants segregated for green and chlorina seedlings in $F_2$, and 22 $F_1$ plants segregated for green, chlorina, and virescent seedlings in $F_2$. The observed ratio as compared with a calculated 9:3:4 ratio is shown in Table 9. The data here given indicate a very poor fit to the calculated 9:3:4 ratio.

TABLE 9.—*Observed and calculated 9:3:4 ratio of green, chlorina, and virescent seedlings in the $F_2$ generation from Coast III×Colsess V*

| Item | Green | Chlorina | Virescent |
|---|---|---|---|
| Observed count | 4,165 | 1,823 | 1,853 |
| Calculated segregation, 9:3:4 | 4,410.5 | 1,470.2 | 1,960.3 |

$\chi^2$=104.1987. $P$, very small.

In order to determine whether linkage was present, Collins's formula

$$p=\sqrt{\frac{AB-2Ab}{AB+Ab}}$$

was used. A crossover percentage of 29.44 was obtained. The observed and calculated ratio on the basis of 29.44 crossing over is given in Table 10. The data here shown indicate that there is a possible linkage between the factor pairs $Y_cy_c$ and $F_cf_c$. While the $P$ value is low, it shows a better fit than that obtained when the observed ratio is tested with a 9:3:4 ratio.

TABLE 10.—*Observed and calculated segregation with 29.44 per cent crossing over in Coast III×Colsess V*

| Item | Green | Chlorina | Virescent |
|---|---|---|---|
| Observed count | 4,165 | 1,823 | 1,853 |
| Calculated segregation, 29.44 per cent crossing over | 4,090.5 | 1,790.3 | 1,960.2 |

$\chi^2$=7.8168. $P$=0.0205.

In order further to test the linkage relationship, the $F_2$ genotypes of the green plants in the $F_2$ families segregating for green, chlorina, and virescent seedlings were determined from $F_3$ seedling counts. Table 11 gives the grouping of 746 $F_2$ plants in the different genotypes, as determined by $F_3$ seedling counts. As in the $F_2$ data, the observed to the calculated 1:2:2:4 ratio for independent inheritance gives a very poor fit.

TABLE 11.—*Grouping of 746 $F_2$ green plants in the different genotypes as determined by $F_3$ seedling counts of their progeny*

| Item | Number of plants showing indicated breeding habit in $F_3$ | | | |
|---|---|---|---|---|
| | Pure green | Green chlorina | Green virescent | Green chlorina virescent |
| Ratio | (a) | 3:1 | 3:1 | 9:3:4 |
| Observed counts | 31 | 145 | 157 | 413 |
| Calculated segregation, 1:2:2:4 | 82.9 | 165.8 | 165.8 | 331.5 |

$\chi^2=55.6058$. *P*, very small.

a All.

The possibility of linkage was calculated from the formula used by Robertson (*10*) and a crossover value of 29.30 was obtained. The observed ratio was compared with the calculated ratio with 29.30 per cent crossing over. Table 12 gives the results obtained when the $\chi^2$ test for goodness of fit is used.

TABLE 12.—*Observed and calculated $F_2$ genotypes of 746 $F_2$ plants obtained from Coast III×Colsess V*

| Item | $F_2$ genotypes of indicated breeding habit | | | |
|---|---|---|---|---|
| | Green | Green chlorina | Green virescent | Green chlorina virescent |
| Observed count | 31 | 145 | 157 | 413 |
| Calculated segregation, 29.3 per cent crossing over | 30.7 | 148.2 | 148.2 | 418.9 |

$\chi^2=0.6777$. *P*, very large.

The fit of the observed to the calculated ratio with 29.30 per cent crossing over is very good, indicating a linkage of the factor pairs $Y_c y_c$ for green versus virescent seedlings in Coast III and the factor pairs $F_c f_c$ for green versus chlorina seedlings in Colsess V. The chlorina factor pair $f_c f_c$ had previously been shown by two of the writers (*11*) to be inherited independently of the factor pairs $A_c a_c$ and $X_c x_c$ found in Colsess and the factor pair $A_t a_t$ for green versus white seedlings found in Trebi. The factor pair *Kk* for hoods versus awns was also found to be inherited independently of the chlorina factor pair. The factor pair $Y_c y_c$ for green versus virescent seedlings in Coast was also reported to be inherited independently of the factor pairs $A_c a_c$, $X_c x_c$, $A_t a_t$, and *Kk*.

GREEN VERSUS CHLORINA SEEDLINGS (Ff) IN MINNESOTA 84–7 AND GREEN VERSUS CHLORINA SEEDLINGS ($F_cf_c$) IN COLSESS

The interrelationship of the factor pairs *Ff* and $F_cf_c$ was studied in a cross between Minnesota 84–7 pure for chlorina (*ff*) and a Colsess plant pure for chlorina ($f_cf_c$). The $F_1$ plants were pure green. The $F_2$ plants segregated into green and chlorina. Table 13 gives the data obtained for several $F_2$ families. The chlorina plants were hard to separate in the field and were grouped together.

TABLE 13.—*$F_2$ segregation of green and chlorina plants from Minnesota 84–7× Colsess V*

| Item | Green | Chlorina | D/PE |
|---|---|---|---|
| Observed count | 4,485 | 3,697 | |
| Calculated segregation, 9:7 | 4,602 | 3,580 | 3.91 |

The number of chlorina plants is larger than the calculated number; the deviation divided by the probable error is 3.91.

The segregation of the green and the two chlorina types was determined from $F_3$ seedling counts. Table 14 gives the $F_2$ segregation of green and the different chlorinas, as determined from the $F_3$ seedling counts. The data here given indicate that the factor pair *Ff* for green and chlorina seedlings in Minnesota 84–7 and the factor pair $F_cf_c$ for green and chlorina in Colsess V are inherited independently of each other. To test further the inheritance of the factor pairs *Ff* and $F_cf_c$, $F_3$ seedling counts were made on the progeny of $F_2$ green plants. With independent inheritance, a ratio of 1 green to 2 segregating for green and chlorina, *Ff*; 2 segregating for green and chlorina, $F_cf_c$; and 4 segregating for green and both chlorinas would be expected. A close approach to a calculated 1:2:2:4 ratio was obtained, $\chi^2 = 1.9345$, which gave a $P$ value of 0.5874. This further indicated that the factor pairs *Ff* and $F_cf_c$ are inherited independently of each other.

TABLE 14.—*$F_2$ classes as determined from $F_3$ seedling counts in Minnesota 84–7× Colsess V*

| Item | Green | Minnesota 84–7 chlorina | Colsess V chlorina | Double chlorina |
|---|---|---|---|---|
| Observed count | 1,158 | 346 | 322 | 120 |
| Calculated segregation for independence | 1,143.8 | 360.2 | 336.2 | 105.8 |

$\chi^2 = 3.2418$. $P = 0.0718$.

GREEN VERSUS XANTHA SEEDLINGS ($X_cx_c$) AND GREEN VERSUS VIRESCENT SEEDLINGS (*Yy*)

Crosses were made between Colsess IV plants heterozygous for yellow seedlings ($X_cx_c$) and Minnesota 72–8 heterozygous for virescent seedlings (*Yy*) (green-tipped whites). Twenty-nine $F_1$ plants were grown. Of these, 11 gave only pure-green progeny, 6 segregated for green and yellow, 6 segregated for green and virescent seedlings, and 6 segregated for green, yellow, and virescent seedlings. The number of green plants was somewhat larger than expected.

INTERRELATIONSHIP OF THE YELLOW AND VIRESCENT SEEDLING FACTORS

The progeny of $F_1$ green plants which segregated for all three types of seedlings in $F_2$ were used. In Table 15 the plants are grouped according to the $F_2$ genotype, as determined from the $F_3$ seedling segregations.

TABLE 15.—*Observed and calculated* $F_2$ *genotypes, as determined by the* $F_3$ *seedling segregations in Colsess IV* × *Minnesota 72–8*

| Item | Number of indicated genotype | | | |
|---|---|---|---|---|
| | $X_cX_cYY$ | $X_cX_cYy$ | $X_cx_cYY$ | $X_cx_cYy$ |
| Observed count | 171 | 352 | 315 | 646 |
| Calculated segregation, 1:2:2:4 | 164.9 | 329.8 | 329.8 | 659.6 |

$\chi^2=2.6647$. $P=0.4522$.

The data in Table 15 indicate that the factor pairs $X_cx_c$ and $Yy$ are inherited independently of each other.

## RELATION OF CHLOROPHYLL DEFICIENCIES TO OTHER BOTANIAL CHARACTERS

### GREEN VERSUS CHLORINA SEEDLINGS (Ff) AND LONG VERSUS SHORT HAIRED RACHILLA (Ss)

The interrelationship of the green versus chlorina seedling color and long versus short haired rachilla was studied in the $F_2$ plants producing only green and chlorina seedlings. Table 16 gives the observed values and the calculated 9:3:3:1 ratio for green versus chlorina seedlings and long versus short haired rachilla.

TABLE 16.—*$F_2$ segregation of green versus chlorina seedlings and long versus short haired rachilla*

| Item | Number of plants having character indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | Long | Short | Long | Short |
| Observed count | 810 | 266 | 275 | 95 |
| Calculated segregation, 9:3:3:1 | 813.4 | 271.1 | 271.1 | 90.4 |

$\chi^2=0.4029$. *P*, very large.

The agreement between the observed and the calculated ratio for independent inheritence is very good and indicates that the factor pairs *Ff* and *Ss* are inherited independently of each other.

### GREEN VERSUS CHLORINA SEEDLINGS (Ff) AND NON 6-ROWED VERSUS 6-ROWED (Vv)

In studying the interrelationship of the non 6-rowed and 6-rowed character pair with green versus chlorina, it was found that the green plants were high in 6-rowed plants and the chlorina plants were high in non 6-rowed plants. The 6-rowed plants had the following genotypes, *vvII*, *vvIi*, and *vvii*. If the non 6-rowed plants are grouped as

2-rowed and intermediate plants, the 2-rowed plants would have the following genotypes, *VVII*, *VViI*, and *VVii*. This type of classification can be made in the $F_2$. If the genotypes of the intermediate classes *VvIi*, *Vvii*, *VvII* are grouped the ratio is as follows: One 6-rowed, two intermediate, and one 2-rowed. The intermediate classes contained both high and low fertility types of intermediates. A check of the $F_2$ counts was made from $F_3$ segregation of $F_2$ plants. Only four changes in classification if 619 $F_2$ plants were necessary. Two 2-rowed plants were changed to an intermedium, one intermediate high-fertility plant to a 6-rowed, and one intermediate to an intermedium. This would only change the place of two plants in the 1:2:1 ratio.

When 2-rowed plants (*VVII*, *VViI*, and *VVii*) are grouped in one class and the non 2-rowed (6-rowed *vvII*, *vvIi*, *vvii*, and intermediate *VvIi*, *Vvii*, *VvII*) are grouped in the other class, a ratio approaching 1 2-rowed to 3 non 2-rowed was obtained. Similarly, when the $F_2$ plants were classified as non 6-rowed (2-rowed *VVII*, *VViI*, *VVii*, and intermediate *VvIi*, *Vvii*, and *VvII*) and 6-rowed (*vvII*, *vvIi*, and *vvii*) a good fit to a 3:1 ratio was obtained.

When the interrelationship of green versus chlorina and non 2-rowed versus 2-rowed is studied, a coupling type of linkage is found. Table 17 gives the $F_2$ segregation of non 2-rowed versus 2-rowed and green versus chlorina plants. The fit of the observed to the calculated is very poor.

TABLE 17.—*$F_2$ segregation of non 2-rowed versus 2-rowed and green versus chlorina plants*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | Non 2-rowed | 2-rowed | Non 2-rowed | 2-rowed |
| Observed count | 1,028 | 118 | 142 | 262 |
| Calculated segregation, 9:3:3:1 | 871.9 | 290.6 | 290.6 | 96.9 |

$\chi^2 = 487.75$. *P*, very small.

The product-moment method was used to determine the crossover percentage between the factor pairs *Vv* and *Ff*. Immer's (*8*) tables were used. A crossover percentage of 18.3 was obtained. Table 18 gives the fit of the observed to the calculated ratio with 18.3 per cent of crossing over.

TABLE 18.—*$F_2$ segregation of non 2-rowed versus 2-rowed and green versus chlorina plants compared with a calculated ratio with 18.3 per cent crossing over*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | Non 2-rowed | 2-rowed | Non 2-rowed | 2-rowed |
| Observed count | 1,038 | 118 | 142 | 262 |
| Calculated segregation, 18.3 per cent crossover | 1,033.6 | 128.9 | 128.9 | 258.6 |

$\chi^2 = 2.3280$. $P = 0.5131$.

Table 18 indicates a linkage of the factor pairs *Ff* and *Vv*, with a crossover value of 18.3 per cent. The probably error for the crossover value is $\pm 0.74$ per cent.

When the non 6-rowed versus the 6-rowed and green versus chlorina plants are tested, there is again a poor fit of the observed to the calculated 9:3:3:1 ratio. The segregation of this type is opposite to the previous classification for rows and is in the repulsion phase, since non 6-rowed and chlorina went into the cross together and 6-rowed and green went into the cross together. The crossover value was again determined by the product-moment method, and a crossover percentage of $16.76 \pm 1.65$ per cent was obtained. Table 19 presents the $F_2$ observed ratio and the calculated ratio with 16.76 per cent crossover. The fit of the observed to the calculated ratio is good in both cases.

TABLE 19.—*$F_2$ segregation of non 6-rowed versus 6-rowed, and green versus chlorina plants compared with a calculated $F_2$ ratio with 16.76 per cent crossing over*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | Non 6-rowed | 6-rowed | Non 6-rowed | 6-rowed |
| Observed count | 783 | 363 | 393 | 11 |
| Calculated segregation 16.76 per cent crossing over | 785.8 | 376.6 | 376.6 | 10.9 |

$\chi^2 = 1.1318$. $P = 0.7711$.

From the data presented in Tables 18 and 19, it may be concluded that the factor pair *Ff* for green versus chlorina seedlings is closely linked to the factor pair *Vv*, which distinguishes the characters 2-rowed and 6-rowed. The crossover value is about $18.3 \pm 0.74$ per cent.

### GREEN VERSUS CHLORINA (Ff) AND HOODS VERSUS AWNS (Kk)

The interrelationship of the green versus chlorina factor pair, *Ff*, and hoods versus awns, *Kk*, was studied in a cross between Colsess and Minnesota 84–7. The $F_2$ segregation of the green versus chlorina plants for hoods versus awns is given in Table 20. The observed ratio fits the calculated 9:3:3:1 ratio very well, $\chi^2$ being 2.5725 with a *P* value of 0.4689. This indicates that the factor pairs *Ff* and *Kk* are inherited independently of each other.

TABLE 20.—*Segregation of green versus chlorina (Ff) plants and hoods versus awns (Kk) in Colsess × Minnesota 84–7*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | Hooded | Awned | Hooded | Awned |
| Observed count | 931 | 326 | 326 | 119 |
| Calculated segregation, 9:3:3:1 | 957.4 | 319.1 | 319.1 | 106.4 |

$\chi^2 = 2.5725$. $P = 0.4689$.

GREEN VERSUS XANTHA SEEDLINGS ($X_cx_c$) AND NONBLUE VERSUS BLUE ALEURONE (Bl bl)

The relationship of the factor pair for green versus xantha seedlings ($X_cx_c$) and the factor pair for nonblue versus blue aleurone (*Bl bl*) was tested in a cross between Colsess IV and Minnesota 72–8. There was no indication of a discrepancy in the 3:1 ratio of plants with nonblue to those with blue aleurone, as is shown in Table 21.

TABLE 21.—*$F_2$ segregation of plants with nonblue and blue aleurone in families which produced xantha seedlings in Colsess IV×Minnesota 72–8*

| Item | Nonblue | Blue | D/PE |
|---|---|---|---|
| Observed count | 2,056 | 704 | |
| Calculated segregation, 3:1 | 2,070 | 690 | 0.91 |

With close linkage of the blue factor and xantha seedlings, there should be a smaller number of plants with blue aleurone, since the factor *Bl* and $x_c$ went into the cross together. No such condition is found, indicating independence of the factor pairs *Blbl* and $X_cx_c$.

GREEN VERSUS CHLORINA SEEDLINGS ($F_cf_c$) AND LONG VERSUS SHORT HAIRED RACHILLAS ($S_s$)

The interrelationship of green versus chlorina seedlings $F_cf_c$ and long versus short haired rachilla *Ss* was studied in a cross between Colsess *V* and Nepal. The $F_2$ segregation is given in Table 22. The data in this table indicate that the factor pairs $F_cf_c$ and *Ss* are inherited independently of each other.

TABLE 22.—*$F_2$ segregation of green versus chlorina ($F_cf_c$) seedlings and long versus short haired rachillas (Ss) in Colsess V×Nepal*

| Item | Number of plants having character indicated | | | |
|---|---|---|---|---|
| | Green | | Chlorina | |
| | S | s | S | s |
| Observed count | 1,392 | 486 | 387 | 119 |
| Calculated segregation for independence | 1,401.4 | 476.6 | 377.6 | 128.4 |

$\chi^2=1.1707$. $P=0.6547$.

## INTERRELATIONSHIP OF OTHER BOTANICAL CHARACTERS

BLUE VERSUS NONBLUE ALEURONE (Bl bl) AND HOODS VERSUS AWNS (Kk)

The interrelationship of the factor pairs *Bl bl* and *Kk* was studied in a cross between Colsess and Minnesota 72–8. The Colsess seeds have a blue aleurone and Minnesota 72–8 a white aleurone. The aleurone color was separated into nonblue and blue, as previously mentioned. Table 23 gives the segregation of nonblue versus blue and hoods versus awns.

TABLE 23.—*Observed and calculated 9:3:3:1 ratio of nonblue versus blue and hoods versus awns in Colsess×Minnesota 72–8*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Nonblue | | Blue | |
| | Hooded | Awned | Hooded | Awned |
| Observed count | 3,086 | 1,334 | 1,455 | 73 |
| Calculated segregation, 9:3:3:1 | 3,345.75 | 1,115.25 | 1,115.25 | 371.75 |

$\chi^2=406.65$. *P*, very small.

The observed ratio fits the calculated 9:3:3:1 ratio very poorly. The two middle classes, however, are noticeably high and the two extreme classes low. Since nonblue went into the cross with awns, and blue went into the cross with hoods, a linkage of the repulsion type might be expected. The segregation of the $F_2$ plants indicates such a linkage. The possible linkage value was calculated by the product-moment method with the use of Immer's tables (*8*), and a crossover value of $22.58 \pm 0.82$ per cent was obtained.

The data in Table 24 indicate a linkage of the factor pairs *Bl bl* and *Kk*. The linkage agrees with the finding of Buckley (*1*). However, he found a crossover value of 40.56. In his studies he used 714 plants.

TABLE 24.—*Observed and calculated ratio of nonblue versus blue and hoods versus awns with 22.58 per cent crossing over*

| Item | Number of plants having character indicated | | | |
|---|---|---|---|---|
| | Nonblue | | Blue | |
| | K | k | K | k |
| Observed count | 3,086 | 1,334 | 1,455 | 73 |
| Calculated segregation, 22.58 per cent crossing over | 3,049.8 | 1,411.2 | 1,411.2 | 75.8 |

$\chi^2=6.1157$. $P=0.1070$.

Similar wide deviations from the calculated 9:3:3:1 ratio were found for the factor pairs *Bl bl* and *Kk* in the families segregating for green and yellow seedlings in the $F_2$ and also in the families segregating for green and virescent seedlings in the $F_2$. As has already been shown, the nonblue and blue segregation of aleurone color both gave good fits to the calculated 3:1 ratio. There is evidently no linkage between the factor pairs $X_c x_c$ for green versus yellow seedlings and $Yy$ for green versus virescent seedlings and aleurone color.

Crossover percentages were calculated for the factor pairs *Bl bl* and *Kk* in families segregating for green and yellow seedlings and families segregating for green and virescent seedlings. The crossover values were $25.01 \pm 1.19$ and $24.70 \pm 1.21$. In the former case 2,760 plants were used and in the latter 2,667.

When the observed and calculated values with 25.01 and 24.70 crossover percentage were tested, a $\chi^2$ of 1.5606 with a $P$ value of 0.6602 was obtained in the families segregating for green and yellow

seedlings, and a $\chi^2$ of 0.5967 with a very large value for $P$ was obtained in the families segregating for green and virescent seedlings. While the latter crossover values are slightly higher than that calculated from the green plants, the difference is within three times the probable error of a difference. The crossover percentage is evidently about 22.00.

BLUE VERSUS NONBLUE ALEURONE (Bl bl) AND LONG VERSUS SHORT HAIRED RACHILLA (Ss)

A total of 2,555 $F_2$ plants was used in this study. A slight deviation in the nonblue and blue segregation was found. The blue-seeded plants were in larger numbers than the calculated 3:1 ratio of nonblue versus blue. In order to overcome the error caused by this discrepancy, the $F_2$ segregation was tested for independence. The results given in Table 25 were obtained.

TABLE 25.—*$F_2$ segregation for nonblue versus blue and long versus short haired rachilla in pure green plants*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Nonblue | | Blue | |
| | Long-haired rachillas | Short-haired rachillas | Long-haired rachillas | Short-haired rachillas |
| Observed count | 1,386 | 471 | 511 | 187 |
| Calculated segregation | 1,378.75 | 478.25 | 518.25 | 179.75 |

$\chi^2$=0.5418. $P$, very large.

These data indicate that the factor pairs *Bl bl* and *Ss* are inherited independently of each other. This, again, agrees with Buckley's findings.

BLACK VERSUS WHITE GLUMES (Bb) AND ROUGH VERSUS SMOOTH AWNS (Rr R′r′)

The interrelationship of the factor pairs *Bb* and *Rr R′r′* was studied in a Coast × Lion cross. Table 26 presents the data obtained in this cross. The calculations were made on a (3:1) (12:3:1) basis. The data indicate that the factor pairs for rough versus smooth awns are inherited independently of the factor pair for black versus white glume color.

TABLE 26.—*$F_2$ segregation for glume color (Bb) and roughness of awn (Rr R′r′) in Coast × Lion*

| Item | Number of plants having characters indicated | | | | | |
|---|---|---|---|---|---|---|
| | Black Glumes | | | White Glumes | | |
| | Rough | Intermediate smooth | Smooth | Rough | Intermediate smooth | Smooth |
| Observed count | 635 | 138 | 49 | 217 | 53 | 15 |
| Calculated segregation (3:1) (12:3:1) | 616.5 | 154.1 | 51.4 | 213.8 | 53.4 | 17.8 |

$\chi^2$=2.85. $P$=0.5845.

BLACK VERSUS WHITE GLUMES (Bb) AND BRANCHED VERSUS UNBRANCHED STYLES (Gg G'g' G''g'')

The interrelationship of the characters glume color and style branching was studied in a (63:1) (3:1) classification of $F_2$ data. Table 27 presents the results obtained.

TABLE 27.—*$F_2$ segregation of style branching (63:1) and glume color (3:1) in Coast × Lion*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Branched style | | Unbranched style | |
| | Black | White | Black | White |
| Observed count | 806 | 282 | 16 | 3 |
| Calculated segregation (63:1) (3:1) | 816 | 272 | 14.25 | 4.75 |

$\chi^2=1.35$. $P=0.5230$.

The data indicate independent inheritance of the factor pairs *Gg G'g' G''g''* and the factor pair *Bb*.

LONG VERSUS SHORT HAIRED RACHILLA (Ss) AND ROUGH VERSUS SMOOTH AWN (Rr R'r')

In studying the interrelationship of long versus short haired rachilla *Ss* and rough versus smooth awn, the factors for the rough-awned character were studied separately. Two types of classification of $F_2$ material were made. One separated the material into rough and smooth. The smooth class included both intermediate smooth and smooth. Table 28 presents the data obtained from the two types of classification. When the $\chi^2$ test for independence was used a very poor fit was obtained.

TABLE 28.—*$F_2$ segregation of long versus short haired rachillas and rough versus smooth awns*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Long-haired rachillas | | Short-haired rachillas | |
| | Rough awns | Smooth awns | Rough awns | Smooth awns |
| Observed count | 593 | 223 | 259 | 32 |
| Calculated segregation | 628 | 188 | 224 | 67 |

$\chi^2=32.2190$. $P$, very small.

In order to determine whether the factor pair *R'r'* was inherited independently of the factor pair *Ss*, which differentiates between long and short haired rachilla, a second type of classification was made in which only the intermediate-smooth and smooth-awned plants were used. The data obtained are given in Table 29.

TABLE 29.—*$F_2$ segregation of long versus short haired rachillas (Ss) and intermediate-smooth versus smooth awns (R′r′)*

| Item | Number of plants having characters indicated | | | |
|---|---|---|---|---|
| | Long-haired rachilla | | Short-haired rachilla | |
| | Intermediate-smooth awns | Smooth awns | Intermediate-smooth awns | Smooth awns |
| Observed count | 482 | 157 | 161 | 60 |
| Calculated segregation, 9:3:3:1 | 483.75 | 161.25 | 161.25 | 53.75 |

$\chi^2=0.8454$. *P*, very large.

The fit of the observed to the calculated is very good, indicating independent inheritance of the factor pairs *R′r′* and *Ss*. These data agree with the findings of Sigfusson (*12*).

Since the factor pair *R′r′* for intermediate smooth versus smooth has been found to be inherited independently of the factor pair *Ss* for long versus short haired rachilla, the intermediate-smooth and smooth phenotypes may be combined in a study of linkage between the factor pairs *Rr* and *Ss*.

According to the symbols used in this paper, the Lion parent has the genetic constitution *rr*, *r′r′*, *SS*, and the Coast parent has the genetic constitution *RR*, *R′R′*, *ss* for the characters roughness of awn and rachilla hairs. The characters went into the cross in the repulsion phase. The dominant factor *R* concerned in this study is in the rough class only, while the recessive factor *r* is in the intermediate-smooth and smooth classes. The factor pair *R′r′* is common to both classes, but should not interfere in the calculations, as it was found to be inherited independently of the factor pair *Ss* for long versus short-haired rachilla. The linkage value was calculated on the basis of a (3:1) (3:1) ratio by the use of Immer's tables (*8*). The observed data are compared with the calculated ratio with a crossover percentage of $34.63 \pm 1.76$. (Table 30.)

TABLE 30.—*Comparison of the observed and the calculated ratios with 34.63 per cent crossing over*

| Item | Number of plants having character indicated | | | |
|---|---|---|---|---|
| | Long-haired rachilla | | Short-haired rachilla | |
| | Rough awns | Intermediate-smooth and smooth awns | Rough awns | Intermediate-smooth and smooth awns |
| Observed count | 593 | 223 | 259 | 32 |
| Calculated segregation, 34.63 per cent crossing over | 576.6 | 239.4 | 256.1 | 34.9 |

$\chi^2=1.86$. $P=0.4004$.

The data in Table 30 indicate a linkage of the factor pairs *Rr* for rough versus smooth awn and *Ss* for long versus short haired rachilla, with a crossover value of $34.63 \pm 1.76$. The above crossover percent-

age agrees fairly well with the crossover percentage of 30.8 reported by Sigfusson (*12*) and of $28.70 \pm 3.43$ in the repulsion phase and $34.54 \pm 2.89$ in the coupling phase reported by Hor (*7*).

### ROUGH VERSUS SMOOTH AWN (Rr R'r') AND BRANCHED VERSUS UNBRANCHED STYLES (Gg G'g' G''g'')

The interrelationship of the factor pairs *Rr R'r'* for rough versus smooth awn and *Gg G'g' G''g''* for branched versus unbranched style was studied in the same cross. Table 31 gives the segregation of rough, intermediate-smooth, and smooth-awned plants with branched and unbranched styles.

TABLE 31.—*$F_2$ segregation of branched versus unbranched styles (Gg G'g' G''g'') and rough versus smooth awns (Rr R'r') in Coast × Lion*

| Item | Number of plants having characters indicated | | | | | |
|---|---|---|---|---|---|---|
| | Branched styles | | | Unbranched styles | | |
| | Rough awns | Intermediate-smooth awns | Smooth awns | Rough awns | Intermediate-smooth awns | Smooth awns |
| Observed count | 850 | 188 | 50 | 2 | 3 | 14 |
| Calculated segregation on a basis of two 12:3:1 | 816 | 204 | 68 | 14.25 | 3.56 | 1.19 |

$\chi^2 = 156.54$. *P*, very small.

The segregation in Table 31 is between a 2-factor difference for roughness of awn and a 3-factor difference for branching of style. The unbranched style group has only 19 plants. However, the unbranched style-smooth awn class is high and the unbranched style-rough-awned class is low when compared with the calculated 12:3:1 ratio. The reverse is true, to a lesser extent, in the branched style group. The above deviation from the calculated 12:3:1 ratio indicates linkage. The dominant factors concerned in this cross enter in the Coast parent, and the recessive factors enter the cross together in the Lion parent. Therefore, if there is linkage of the factor pairs for branched style and rough awn or unbranched style and smooth awn, it would be in the coupling phase. If such a linkage occurred, the rough-awned plants with branched styles and the smooth-awned plants with unbranched styles would be present in greater numbers than would be expected with independent inheritance. Similarly, the rough-awned plants with unbranched styles and the smooth-awned plants with branched styles would be less numerous than the calculated ratio for independent inheritance. Such a condition is found in Table 31, indicating a possible linkage between the main factor pair for rough awns (*Rr*) and one of the factor pairs for style branching. The data, however, are not sufficient to permit the calculation of the crossover percentage with any degree of accuracy.

## DISCUSSION

In studying linkage relationships, only those having a direct bearing on the factor pairs considered in this paper will be discussed. A rather extensive list of linkage relationships has recently been made by Daane (*2*) and Buckley (*1*).

GROUP 1: NON 6-ROWED VERSUS 6-ROWED

The following plant characters have been described by two or more independent workers as belonging to this group (*2*): Height of plant, length of awn, early versus late heading, and extension of the outer glume.

In crosses between Minnesota 84–7 and Trebi, a linkage has been found between the factor pair *Vv*, which distinguishes between the 2-rowed and 6-rowed character, and *Ff*, a factor pair for chlorina seedlings first described by Nilsson-Ehle (*9*) and later obtained from the Minnesota station under the number Minnesota 84-7. This chlorina seedling was found by Nilsson-Ehle to be linked with a white seedling factor pair known as $A_3a_3$, albino 3. Hallquist (*4*) confirmed this linkage and found the linkage of another white seedling factor pair, $A_4a_4$, albino 4. He gave the following crossover percentages and arrangement of the genes in the chromosome: Albino 3 and chlorina, 10.2 per cent crossover; albino 4 and chlorina, 3.8 per cent crossover; and albino 3 and albino 4, 12.5 per cent crossover. This would mean that the genes were arranged in the chromosome as follows: Albino 4, chlorina and albino 3. Buckley (*1*) in a recent paper reported a linkage of the factor pair for 2-rowed versus 6-rowed, with several genes concerned in the development of colored veins on the lemma and one of the two genes concerned with the development of red pericarp. He also lists the chlorophyll-deficient series of Nilsson-Ehle and Hallquist as possibly forming a fifth linkage group. The data presented in this paper place this group in the linkage group with 6-rowed versus 2-rowed. However, the arrangement of the factors in the chromosome has not yet been determined. The factor pair for the row character may be either to the right or to the left of the factor pair for chlorina.

GROUP 2: BLACK VERSUS WHITE LEMMA AND PERICARP

Several factor pairs are located in this group, but none so far has been found independently by two workers. However, it has been clearly shown by Robertson (*10*), Sigfusson (*12*), and Buckley (*1*) that the factor pair *Ss*, for long versus short-haired rachilla is inherited independently of the factor pair *Bb* for black versus white pericarp.

GROUP 3: HULLED VERSUS NAKED CARYOPSIS

Only one factor pair has been reported by two workers in this group that is dense versus lax head. (*2*).

GROUP 4: HOODS VERSUS AWNS

Buckley found a linkage between the factor pairs for hoods versus awns and *Bl bl* for blue versus nonblue aleurone. This linkage is confirmed in the present paper. However, the linkage value here reported is smaller than that found by Buckley (*1*). It was also shown that the chlorophyll-deficiency factor pairs $A_cA_c$ and $X_cX_c$ which are closely linked and which were thought to be loosely linked to the factor pair *Kk* for hoods versus awns, are evidently not linked to the factor pair *Bl bl* for blue versus nonblue aleurone. This is easily understood when it is remembered that the crossover value reported by Robertson (*10*) from a segregation of three classes was 45.09 per cent. If *Kk* was also linked with $A_cA_c$ a linkage of $A_cA_c$ and *Bl bl* should have been obtained with a crossover value of about 25 per cent.

GROUP 5: ROUGH VERSUS SMOOTH AWNS

Several workers (*12, 7*) have reported the linkage of one of the factor pairs for rough versus smooth awn (*Rr*) and long versus short haired rachilla (*Ss*). This linkage is confirmed and the linkage values of all three studies are within three times their probable errors. The crossover percentage is about 34.

GROUP 6: ALBINO ($A_cA_c$) VERSUS GREEN

Only one linkage in this group has been found, that of the factor pair $X_cx_c$ for green versus xantha seedlings. The factor pair $A_cA_c$ is not closely linked with the following factors found in the abovementioned groups: (1), Non 6-rowed versus 6-rowed; (2), black versus white lemma; (3), hulled versus naked caryopsis; (4), hoods versus awns; and (5), rough versus smooth awn.

In making the studies reported in this paper, $F_3$ data have been used frequently. The possibility of obtaining coupling phases from $F_2$ and $F_3$ segregations when the $F_2$ 3 to 1 ratios can be separated into 1:2:1 ratios by the use of $F_3$ data is discussed in this paper. When one or other of the factor pairs can be segregated into three classes where a single-factor difference determines the character difference, a 1:2:1 ratio may be obtained and by proper grouping of the heterozygous class with one or the other of the homozygous classes, a 3:1 or 1:3 ratio may be obtained and a coupling phase in the $F_2$ used for the determination of linkage. This method cuts down the error, since the small double recessive group obtained in the repulsion phase of linkage is combined with the heterozygous individuals in the coupling phase and the loss of a few individuals of this class has less influence on the linkage determinations when in the coupling phase. An example of this type of classification with the same data is given in Tables 18 and 19, where the interrelationship of 2 row versus non 2 row and green versus chlorina (*Ff*) is studied.

## SUMMARY

In this paper the inheritance of the following character pairs is explained on a simple Mendelian basis: Green versus chlorina seedlings (*Ff*) in Minnesota 84–7; green versus virescent seedlings (*Yy*) in Minnesota 72–8; blue versus nonblue aleurone (*Bl bl*).

A 2-factor difference was found to explain the difference between rough and smooth awns. A 12:3:1 ratio of rough, intermediate-smooth, and smooth-awned plants was found. The symbols *Rr R′r′* were used.

The interrelationship of several botanical characters and chlorophyll deficiencies was studied, with the following results:

(1) The factor pair *Ff* for green versus chlorina seedlings was found to be inherited independently of the factor pairs $A_tA_t$ for green versus white seedlings in Trebi, $A_cA_c$ for green versus white seedlings in Colsess, $F_cf_c$ for green versus chlorina seedlings in Colsess, *Ss* for long versus short haired rachilla, and *Kk* for hoods versus awns.

(2) The factor pair $X_cx_c$ for green versus xantha seedlings in Colsess was found to be inherited independently of the factor pairs *Yy* for green versus virescent seedlings in Minnesota 72–8 and *Bl bl* for nonblue versus blue aleurone.

The factor pair *Bl bl* for blue versus nonblue aleurone was found to be inherited independently of the factor pair *Ss* for long-haired versus short-haired rachilla.

The factor pair *Bb* for black versus white glumes was inherited independently of the factor pairs *Rr R′r′* for rough versus smooth awn and *Gg G′g′ G″g″* for branched versus unbranched style. The factor pair *Ss* for long versus short haired rachilla seemed to be inherited independently of the factors for branched and unbranched style. This was explained on the hypothesis that the factors may be located at the extreme ends of the chromosome.

Linkage was found between the following factor pairs:

(1) *Ff* for green versus chlorina seedlings and *Vv* for non 6-rowed versus 6-rowed. A crossover value of 18.3 ±0.74 per cent was found.

(2) $F_c f_c$ for green versus chlorina seedlings in Colsess and $Y_c y_c$ for green versus virescent seedlings in Coast. A crossover value of 29.3 per cent was found.

(3) *Bl bl* for blue versus nonblue aleurone and *Kk* for hoods versus awns. The crossover value was 22.58 per cent ±0.82.

(4) *Ss* for long-haired versus short-haired rachilla and *Rr* the main factor pair for roughness of awn. The crossover percentage was 34.63.

(5) There was also an indication of possible linkage between the rough-awn factor pair and some of the factors for branched and unbranched style.

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# A PHOTOGRAPHIC LIGHT BOX FOR USE IN AGRICULTURAL RESEARCH [1]

By A. B. GROVES

*Assistant Plant Pathologist, Department of Botany and Plant Pathology, Virginia Agricultural Experiment Station*

## INTRODUCTION

The difficulty experienced in attempts to obtain a constant and always uniform source of light for photographing diseased fruits and similar specimens led the writer to devise a more satisfactory method of illumination. The result of considerable experimenting was the production of a light box which has proved to be most satisfactory for its purpose, and its use has resulted in the saving of considerable time and material, as well as in obtaining better results than had been possible before. This light box has also proved to be useful in lantern-slide production, natural-color photography, and in low-power photomicrographic work where upper-field illumination is desired.

## APPARATUS

The illuminating device as designed for use with a Leitz vertical camera (pl. 1, A) consists essentially of a box, square at the top and rectangular at the base, with the lower portion of two sides extended 3 inches at one end to make this part of the box project beyond the square upper part. The box is provided with a removable toppiece carrying a shielded aperture for the camera lens. The upper section of the box carries four 50-watt light bulbs which serve as a source of upper illumination, while two bulbs of equal size at the bottom of the box provide illumination from beneath the subject. The projecting right end of the box is hinged to provide ready access to the interior of the apparatus so that the specimen-supporting fixtures may be manipulated and the specimens arranged during the focusing process. Four grooves are cut into the inside faces of each of the two large sides of the box, and the specimen-supporting fixtures are carried in these grooves. These four grooves make it possible to adjust the distance of the subject from the lens and thus obtain a suitable magnification without moving the lens more than a slight amount, if at all. This is important, as the field becomes restricted if the lens is moved any considerable distance upward. The lights are controlled by three switches, one line switch in the cord a short distance from the box and two tumbler switches attached to the outside of the box. The upper switch controls the four bulbs on the upper circuit, and the lower switch controls the lower two bulbs. The line switch is used to control the lights while a plate is being exposed, the other two being manipulated only when the line switch is off. This precaution is necessary to prevent any vibration of the subject while it

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is being photographed. Three pieces of accessory apparatus (pl. 1, D) are used to support the objects to be photographed; two of these are used when a photograph with either a white or gray background is desired, and the third is used only when a completely black background is wanted. The first of these is a 4-sided reflector apparatus (*b*) which carries a sheet of flashed opal glass at its base and a second sheet of frosted glass over this. The flashed opal glass serves to give satisfactory diffusion of the light from below, but because of its smooth surface it must be covered with a sheet of frosted glass to eliminate objectionable glare from the lights. The second equipment is used when larger objects are to be photographed, or when lantern slides are to be made. This equipment consists of two large sheets of flashed opal and frosted glass (*b*) cut to fit directly into the grooves in the sides of the box. It is not altogether necessary to use the tray at any time, as these two large sheets of glass may be employed for the same purposes for which the tray is used. The greater convenience of the tray, together with some advantage in illuminating the sides of deep specimens, seems, however, to make its use desirable. A deep, black-velvet-lined box which fits directly into the side grooves serves as a support when a black background is desired.

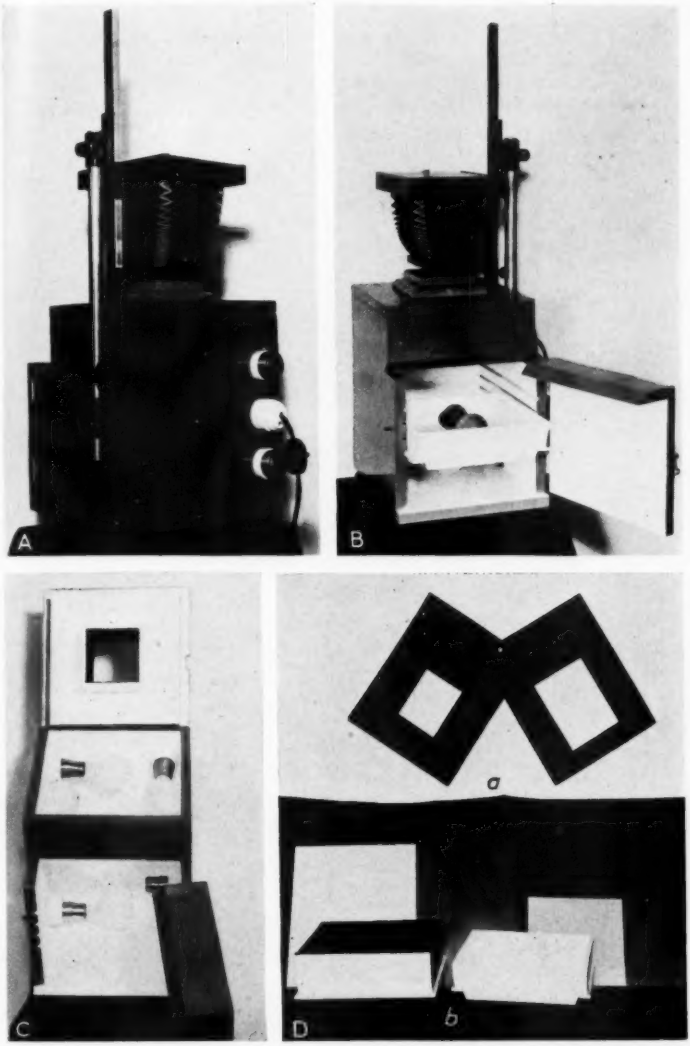
## METHOD OF OPERATION

All of the more common types of photographic plates have been used with the apparatus, but the ones most generally satisfactory have been those with panchromatic emulsions. Orthochromatic plates also give good results, but require several times the exposure necessary for panchromatic plates. The process panchromatic plate is also quite useful where it is desired to accentuate the contrast. Filters have not been used as much as with daylight, but it has been found that the Wratten K 2 and K 3 filters give no appreciable correction. Difficulty was experienced in obtaining the desired contrasts with such subjects as apple and peach leaves and fruits showing spray-injured or diseased regions in which the necrotic or chlorotic areas were light brown, red, or yellow. The use of the proper filter as determined by observation of the object through a filter test chart resulted in securing satisfactory photographs. The Wratten A, B, and G filters are used where it is desired to obtain clear definition with such objects having slight contrasts between greens, reds, brown, and yellow.

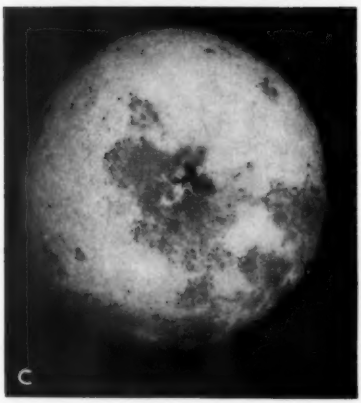
The 100-mm lens is used for practically all the work, as it will cover a 5 by 7 inch plate at the distance it must work from the objects in the box. This lens gives natural-size reproduction as well as a certain degree of enlargement or reduction. The shorter focal-length lenses will not cover as large a field in the necessary working range.

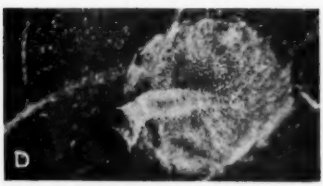
### PHOTOGRAPHING WITH A WHITE BACKGROUND

The white background, as used with the apple target-spot material illustrated (pl. 2, B), is the most generally satisfactory and the most commonly used. It may be employed with any opaque or nearly opaque object, such as fruits, twigs, leaves, tubers, roots, and similar specimens, unless they are very light colored. The object to be photographed is placed directly on the frosted glass (pl. 1, B), which may




Photographic light box and accessories. A, Photographic light box in position, showing manner of fitting vertical camera support and the switch arrangement; B, interior view of box showing the manner of placing specimens for photographing; C, interior view of light box, showing the arrangement of light bulbs; D, accessories for use with light box; the masks for making lantern slides are shown in *a*, while the velvet-lined box, the reflector tray, and the pieces of flashed opal and frosted glass appear in *b*




Photographs made with the aid of the light box, illustrating the different types of background obtainable, and also showing the possibilities of the light box in photomicrography. A, *Lepiota naucina* on black background; B, target canker on apple twigs, white background; C, spray injury on apple, gray background; D, young larva of *Cydia pomonella*. D is × 25

be either the one used in the reflector tray or the large glass. The flashed opal glass must be placed beneath the frosted glass to obtain even illumination from below. The upper, or both the upper and lower, lights are switched on and the arranging of the specimens and the focusing of the camera completed. A diaphram aperture of approximately f. 48 is usually used for the exposure. The lights are switched off, the plate holder inserted, and the protecting slide withdrawn. The lower switch is set at "on," the upper one at "off," and the line switch turned on for approximately two seconds, provided a panchromatic plate without a filter is used. The line switch is then turned off, the upper switch also set at "on," and a second exposure with both sets of lights for approximately one second is given. This completes the exposure. Opaque objects, or those nearly so, may be silhouetted with the lower lights and a small lens diaphram, insuring a satisfactory white background as well as destroying any background shadows. With many objects it is not necessary to use the additional background exposure. In such cases both sets of lights are used together for the single exposure. The periods of exposure vary but little, and consequently after they have once been determined for a certain plate and developer, a high percentage of satisfactory plates may be expected.

### PHOTOGRAPHING WITH A GRAY BACKGROUND

Light to medium gray backgrounds may be produced by inserting a sheet of transparent red paper between the frosted and flashed opal glasses and proceeding in about the same manner as for a white background. A medium-gray background may be obtained with the use of the upper set of lights only; the use of the lower set of lights for varying periods will produce lighter-gray backgrounds. The red paper used in wrapping film packs and other photographic materials is quite satisfactory. The dark-gray background, as used in photographing the apple injured by summer-oil spraying (pl. 2, C), was satisfactorily produced by inserting a sheet of black paper between the two sheets of glass and using only the upper set of lights. The rough surface of the glass will reflect sufficient light to give a gray background, and at the same time no photographic impression of the paper will be obtained as would be the case if the subject were placed directly upon the paper. The gray background is most useful with objects which contain considerable contrast, making either the white or black backgrounds somewhat unsatisfactory.

### PHOTOGRAPHING WITH A BLACK BACKGROUND

Completely black backgrounds are desirable only when sharp contrast is desired, as was the case with the mushroom, *Lepiota naucina*. (Pl. 2, A.) Such a background is obtained by the use of the box lined with black velvet. The subject to be photographed may be placed directly upon the bottom of the box, or upon some small support which is covered by the object itself. Care must be taken to remove all lint and light-colored particles from the surface of the velvet. The upper set of lights are used alone and the usual exposure given. If these precautions are observed an even black background, free from evidences of the support, will be obtained.

### NATURAL-COLOR PHOTOGRAPHY

The Autochrome or Agfa color plates may be used with the light box for taking photographs in natural colors. The writer has not found it necessary to use a filter, the light from the vacuum bulbs producing very satisfactory results without correction. Nitrogen-filled tungsten bulbs would no doubt be desirable if a great deal of this kind of work were to be done. The period of exposure ranges from 20 seconds to over a minute, depending upon the density of plate desired.

### LANTERN-SLIDE PRODUCTION

The light box may be used in making lantern slides from plates not larger than 5 by 7 inches in size. For this purpose a sheet of black cover-stock paper is cut to the size of the large pieces of glass and a correctly centered section slightly smaller than the plate with which it is to be used is cut from the sheet. (Pl. 1, D, *a*.) The section removed must be centered beneath the camera lens. This paper mask is placed between the two large sheets of glass, the three are then slid into two of the parallel grooves which run horizontally between the upper and lower sets of light bulbs (pl. 1, B and C), and the negative placed over the aperture in the paper. The lower lights are switched on and focusing completed, after which the exposure is made in the usual manner.

### PHOTOMICROGRAPHIC ILLUMINATION

The light box may be used quite successfully for upper-field illumination in low-power photomicrographic work (pl. 2, D) when other and more convenient methods are not available. To use the box for this purpose, the lower bulbs are removed and the microscope is placed on the bottom of the box and centered. The tube should be extended to the proper length and the top of the box replaced. The microscope may be too low, in which case it may be raised by placing as many sheets of cardboard beneath it as are necessary to raise it to the desired height. The camera is then fitted to the microscope as usual, the upper lights turned on, and the microscope focused. It is necessary under these circumstances to manipulate the microscope controls through the open door of the box. An exposure of approximately 30 seconds is required at a magnification of 25 diameters. The light box illuminates the field evenly and produces no shadows, shading of the field being necessary if some shadows are desired with light objects showing little contrast.

## CONSTRUCTION AND FITTING OF THE BOX

The light box is not difficult to construct, and may be made by any woodworking or cabinet shop. The total cost, including labor and materials, should not exceed $20 or $25.

Three-quarter-inch white-pine lumber is used in the construction of the box. This makes a very substantial piece of equipment and allows sufficient thickness to cut the wiring and slide grooves. Detailed drawings of all the parts of the box are given in Figures 1–3. The drawings and measurements are based on butt-end construction, although mitered construction may be used if preferred. The ⅜-inch grooves at the top and bottom of the individual pieces, together with

the connecting groove in the left end, are designed to hold the wiring system, which is completely covered in the finished box. These grooves fit together to make continuous channels when the pieces are assembled. The four ¼-inch grooves in each of the two sidepieces are for holding the accessory fittings, as has been previously mentioned.

The back, front, and left sidepieces are nailed to the bottom through the sides, as the bottom fits inside the box and even with the lower

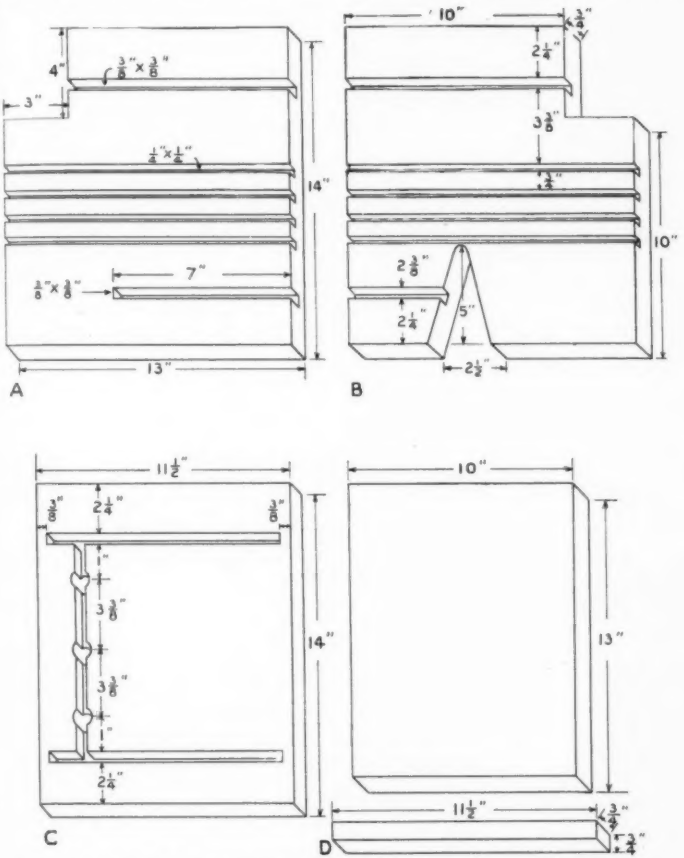



FIGURE 1.—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box: A, Front; B, back; C, left side; D, bottom

edges of the side and end pieces. The upper piece of the right-end assembly is nailed across the upper inset of the two sidepieces. The other two right sidepieces are nailed together as illustrated in Figure 2, B, to form the door of the box. This door is hinged to the side away from the operator, or to the sidepiece designed to fit up against the upright support of the camera. It should be mentioned here that it will be necessary to cut a groove in the outside face of the sidepiece

fitting against the camera support in order to center the camera properly. This groove extends upward from the apex of the triangular cut in the bottom of the sidepiece, but this groove has not been illustrated as the fitting will have to be made to the individual camera. (Pl. 1, A.) The small ¾ by ¾ by 11½ inch piece is nailed across the right end to the bottom piece and extends the bottom out even with the outer edge of the door. When assembled, the inside dimensions

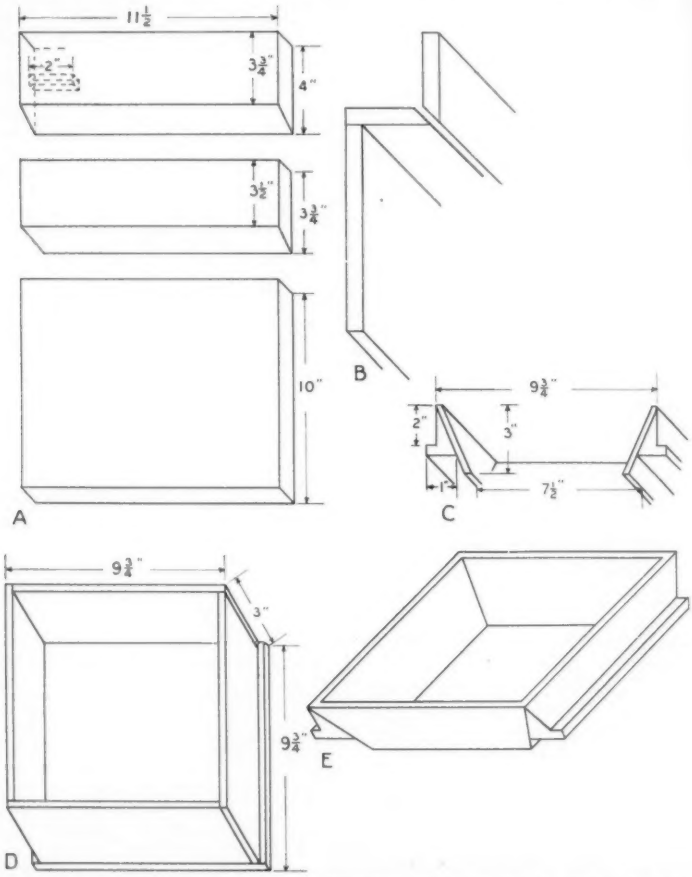



FIGURE 2.—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box: A, Right side; B, detail of right-side construction; C, cross section of the reflector; D, black-background box; E, reflector tray

of the box are 10 by 10 inches at the top and 10 by 13 inches at the bottom. The outside dimensions are 11½ by 14½ inches. The top-piece is 11½ inches square, having a section 4½ by 4½ inches cut from the center. A square tube constructed of ¼-inch plywood is fitted in the hole in the top so that it projects 2¾ inches below the lower surface

and 1 inch above the upper surface of the toppiece. Three-quarter-inch quarter-round molding is fitted around the top of the tube to brace it and give the top a finished appearance. The lower side of the toppiece is fitted with a flange made of triangular stock 1½ inches on each of two sides. (Pl. 1, C, and fig. 3, B.) This piece holds the top

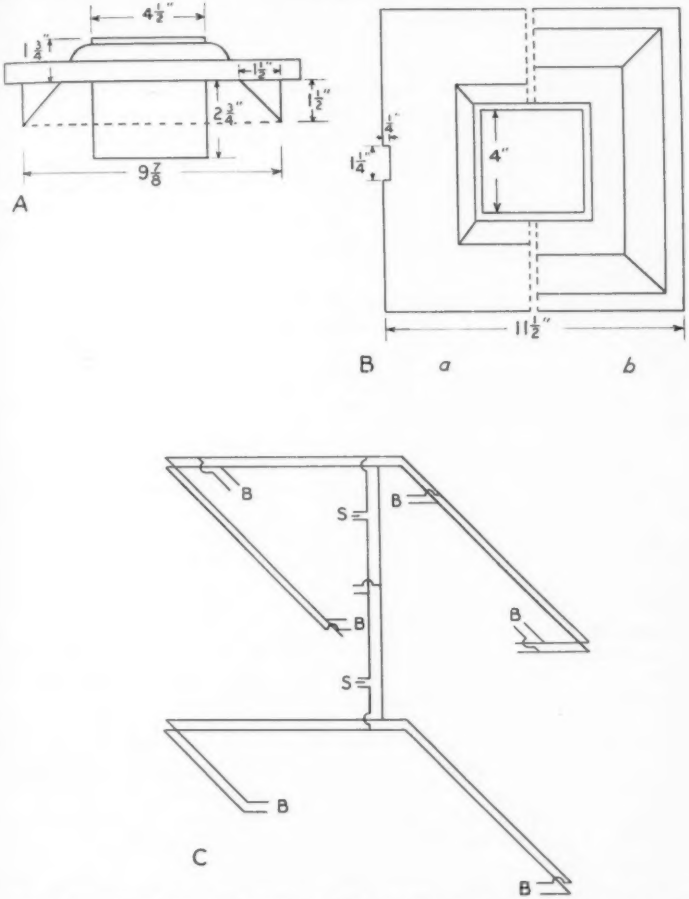



FIGURE 3.—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box, and the arrangement of the light bulbs: A, Cross-sectional view of the top; B, toppiece with upper side shown at *a* and lower side at *b*; C, diagram of wiring in which B stands for bulb and S for switch

in position and also serves as a reflector above and back of the bulbs. The toppiece should not fit too tightly or it may bind after painting.

The box is wired with double-strand insulated wire, which is placed around in the wiring grooves in the box and secured with insulated staples. A loop of wire should be left about 1 inch from each corner

and at the ends of the two lower grooves for later attachment to the light sockets. A single loop is pulled through the upper and lower holes in the left end piece for attachment to the two circuit switches. A double loop is pulled through the center hole to be attached to the rosette to which the lead-in wire is attached. After the wiring has been completed, but before any fixtures have been attached, the wiring grooves are filled with plastic wood material and this allowed to dry thoroughly and harden. The small irregularities remaining are then smoothed over with a thin paste of plaster of Paris and after this has dried and again been smoothed down, the box is ready to be painted. After painting, open-bottom receptacles are fitted at the bulb locations and the wiring brought up through them and attached to the keyless sockets. This assembly of bottom-wired receptacles and keyless sockets places the filaments of the bulbs at approximately the centers of the sides. The switches, rosette, and hinges are also attached after painting has been completed. The arrangement of the bulbs and switches is shown in Plate 1, A and C. The wiring diagram is given in Figure 3, C.

The interior of the box is painted white, as is the reflector tray. Lacquer has proved to be better than enamel for this purpose. The interior of the square lens-receiving tube or aperture is lined with black velvet, although dull black paint should be satisfactory.

The reflector tray is made of ¼-inch plywood, as is the black background box. The reflector tray is constructed with sloping sides, the dimensions at the top being 9¾ by 9¾ inches and 7½ by 7½ inches at the bottom. The tray has no other bottom than the two pieces of glass which serve as a transparent base. The two pieces of glass are cut 8 inches square, thus fitting near the bottom of the tray. The tray is fitted with projecting tongues on two sides (fig. 2, C and E) to fit into the side grooves. The black background box is a plain open-top box 9¾ inches square and 3 inches deep (fig. 2, D) with the bottom edge projecting a short distance on each of two sides in order to engage in the grooves in the box. The interior of the box is lined with a good grade of black velvet. This box may also be used quite satisfactorily outside the box. The two large pieces of frosted and flashed opal glass are each cut 10⅜ by 13 inches in size.

The light box as described is designed to operate with the Leitz vertical camera, although the design may be readily adapted to other cameras. The principal precautions to observe are to see that the over-all height of the box is not greater than the height to which the lower end of the camera rail can be raised, and to determine the largest size box that can be centered beneath the camera.

## SUMMARY

The light box described in this paper has proved very satisfactory as a source of illumination for photographing diseased fruits and similar specimens. It provides a simple and inexpensive means by which shadows, high lights, and cross lights can be eliminated and at the same time enables the operator to secure the color of background that is best suited for the object to be photographed.

Since the illumination comes entirely from artificial sources, daylight being excluded, the light is always uniform. This makes it possible for one to do photographic work at any time of day or at

night, and so long as other factors are kept the same the period of exposure for best results will not vary appreciably. This results in a great saving in time and photographic supplies.

In addition to being of use in ordinary indoor photography, the box may be used for making lantern slides and in low-power photomicrographic work. The construction of the box, together with its method of operation, is described in detail.

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